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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68 — A2

(11) International Publication Number: WO 98/20162

(43) International Publication Date: 14 May 1998 (14.05.98)

(21) International Application Number: PCT/US97/20014

(22) International Filing Date: 5 November 1997 (05.11.97)

(30) Priority Data:

08/743,798	5 November 1996 (05.11.96)	US
60/040,155	7 March 1997 (07.03.97)	US
08/873,597	12 June 1997 (12.06.97)	US
08/873,978	12 June 1997 (12.06.97)	US
08/899,510	24 July 1997 (24.07.97)	US
08/911,085	14 August 1997 (14.08.97)	US
08/911,589	14 August 1997 (14.08.97)	US

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: ELECTRODES LINKED VIA CONDUCTIVE OLIGOMERS TO NUCLEIC ACIDS

(57) Abstract

The invention relates to nucleic acids covalently coupled to electrodes via conductive oligomers. More particularly, the invention is directed to the site-selective modification of nucleic acids with electron transfer moieties and electrodes to produce a new class of biomaterials, and to methods of making and using them.

ELECTRODES LINKED VIA CONDUCTIVE OLIGOMERS TO NUCLEIC ACIDS

FIELD OF THE INVENTION

The invention relates to nucleic acids covalently coupled to electrodes via conductive oligomers. More particularly, the invention is directed to the site-selective modification of nucleic acids with electron transfer moieties and electrodes to produce a new class of biomaterials, and to methods of making and using them.

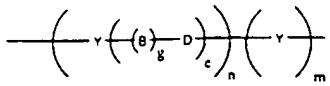
BACKGROUND OF THE INVENTION

The detection of specific nucleic acids is an important tool for diagnostic medicine and molecular biology research. Gene probe assays currently play roles in identifying infectious organisms such as bacteria and viruses, in probing the expression of normal genes and identifying mutant genes such as oncogenes, in typing tissue for compatibility preceding tissue transplantation, in matching tissue or blood samples for forensic medicine, and for exploring homology among genes from different species.

Ideally, a gene probe assay should be sensitive, specific and easily automatable (for a review, see Nickerson, Current Opinion in Biotechnology 4:48-51 (1993)). The requirement for sensitivity (i.e. low detection limits) has been greatly alleviated by the development of the polymerase chain reaction (PCR) and other amplification technologies which allow researchers to amplify exponentially a specific nucleic acid sequence before analysis (for a review, see Abramson et al., Current Opinion in Biotechnology, 4:41-47 (1993)).

Specificity, in contrast, remains a problem in many currently available gene probe assays. The extent of molecular complementarity between probe and target defines the specificity of the interaction. Variations in the concentrations of probes, of targets and of salts in the hybridization medium, in the reaction temperature, and in the length of the probe may alter or influence the specificity of the probe/target interaction.

It may be possible under some limited circumstances to distinguish targets with perfect complementarity from targets with mismatches, although this is generally very difficult using traditional technology, since small variations in the reaction conditions will alter the hybridization. New experimental techniques for mismatch detection with standard probes include DNA ligation assays



wherein

Y is an aromatic group;

n is an integer from 1 to 50;

g is either 1 or zero;

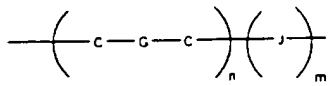
e is an integer from zero to 10;and

m is zero or 1;

wherein when g is 1, B-D is a conjugated bond; and

wherein when g is zero, e is 1 and D is preferably carbonyl, or a heteroatom moiety, wherein the heteroatom is selected from oxygen, sulfur, nitrogen, silicon or phosphorus.

In an additional aspect, the conductive oligomer has the formula:



wherein

n is an integer from 1 to 50;

m is 0 or 1;

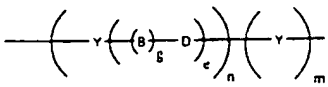
C is carbon;

J is carbonyl or a heteroatom moeity, wherein the heteroatom is selected from the group consisting of oxygen, nitrogen, silicon, phosphorus, sulfur; and

G is a bond selected from alkane, alkene or acetylene.

In a further aspect, the invention provides methods of detecting a target sequence in a nucleic acid sample. The method comprises applying a first input signal to a hybridization complex and detecting electron transfer. The hybridization complex comprises the target sequence, if present, and at least a first probe nucleic acid. The probe nucleic acid comprises a a covalently attached conductive oligomer. The conductive oligomer is also covalently attached to a first electron transfer moiety comprising an electrode. In addition, the hybridization complex has a covalently attached second electron transfer moiety.

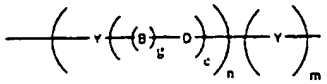
In one aspect, the conductive oligomer has the formula:



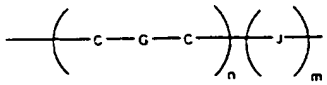
or

J is carbonyl or a heteroatom moeity, wherein the heteroatom is selected from the group consisting of oxygen, nitrogen, silicon, phosphorus, sulfur; and
G is a bond selected from alkane, alkene or acetylene, wherein if m = 0, at least one G is not alkane.

In an additional aspect, the invention provides compositions comprising (a) a solid support comprising a monolayer of passivation agent; (b) a nucleic acid comprising at least one nucleoside, wherein said nucleic acid is covalently attached to said solid support with a linker selected from the group selected from:



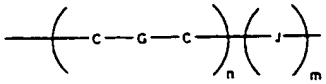
or



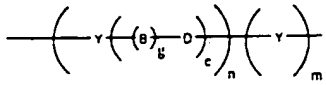
wherein
n is an integer from 1 to 50;
m is 0 or 1;
C is carbon;
J is carbonyl or a heteroatom mpeity, wherein the heteroatom is selected from the group consisting of oxygen, nitrogen, silicon, phosphorus, sulfur; and
G is a bond selected from alkane, alkene or acetylene, wherein if m = 0, at least one G is not alkane

In an additional aspect, the invention provides compositions comprising (a) an electrode; (b) at least one metallocene; and (c) a conductive oligomer covalently attached to both said electrode and said metallocene, wherein said conductive oligomer is selected from the group consisting of:

i)



or



In a further aspect, the invention provides peptide nucleic acids with at least one chemical substituent covalently attached to the α-carbon of a subunit of the peptide nucleic acid.

In an additional aspect, the invention provides peptide nucleic acids with at least one chemical substituent covalently attached to an internal subunit of the peptide nucleic acid.

Figures 9A and 9B depict the response with varying frequency. Figure 9A shows overlaid voltammograms of an electrode coated with a ferrocene-conductive oligomer model complex (wire-2). Four excitation frequencies were applied, 10 Hz, 100 Hz, 1 kHz and 10 kHz, all at 25 mV overpotential. Again, current increases with frequency. Figure 9B shows overlaid voltammograms of electrodes coated with either ssDNA or dsDNA. ssDNA was run at 1 Hz and 10 Hz at 100 mV overpotential (bottom two lines). dsDNA was run at 1, 10, 50 and 100 Hz at 10 mV overpotential (top four lines). Note that the scales between Figure 8 and Figures 9A and 9B are different.

Figure 10 depicts the frequency response of these systems. The peak currents at a number of frequencies are determined and plotted. Sample 3 (filled triangles) responds to increasing frequencies through 10 kHz (system limit), while samples 1 (open circles) and 2 (filled circles) lose their responses at between 20 and 200 Hz. This data was not normalized to the increase in current associated with increasing frequency.

Figure 11 depicts the frequency responses of ssDNA (open circles; sample 5) and dsDNA (filled circles; sample 6) at 25 mV overpotential. The current has been normalized. The curves are not a fit to the data; rather, these are models of RC circuits, illustrating that the data can be fit to such curves, and that the system is in fact mimic standard RC circuits. The top curve was modeled using a 500 ohm resistor and a 0.001 farad capacitor. The bottom curve was modeled using a 20 ohm resistor and a 0.002 farad capacitor.

Figure 12 shows that increasing the overpotential will increase the output current.

Figures 13A and 13B illustrate that the overpotential and frequency can be tuned to increase the selectivity and sensitivity, using Sample 1.

Figure 14 shows that ferrocene added to the solution (Sample 7; open circles) has a frequency response related to diffusion that is easily distinguishable from attached ferrocene (Sample 3; filled circles).

Figures 15A and 15B shows the phase shift that results with different samples. Figure 15A uses two experiments of Sample 1, Sample 3 and Sample 4. Figure 15B uses Sample 5 and Sample 6.

Figure 16 depicts the synthetic scheme for a conductive oligomer covalently attached to a uridine nucleoside via an amine bond, with a CH2 group as a Z linker. Compound C4 can be extended as outlined herein and in Figure 1.

Figure 29 depicts the synthetic scheme for a PNA monomeric subunit with a ferrocene covalently attached to a uracil base, for incorporation into a growing PNA.

Figure 30 depicts the synthetic scheme for a three unit conductive oligomer covalently attached to a base of a PNA monomeric subunit.

Figure 31 depicts the synthetic scheme for a three unit conductive oligomer covalently attached to the backbone of a PNA monomeric subunit.

Figure 32 depicts the synthetic scheme for a ferrocene covalently attached to the backbone of a PNA monomeric subunit.

DETAILED DESCRIPTION OF THE INVENTION

The present invention capitalizes on the previous discovery that electron transfer apparently proceeds through the stacked π-orbitals of the heterocyclic bases of double stranded (hybridized) nucleic acid ("the π-way"). This finding allows the use of nucleic acids containing electron transfer moieties to be used as nucleic acid probes. See PCT publication WO 95/15971, hereby incorporated by reference in its entirety, and cited references. This publication describes the site-selective modification of nucleic acids with redox active moieties, i.e. electron donor and acceptor moieties, which allow the long-distance electron transfer through a double stranded nucleic acid. In general, electron transfer between electron donors and acceptors does not occur at an appreciable rate when the nucleic acid is single stranded, nor does it occur appreciably unless nucleotide base pairing exists in the double stranded sequence between the electron donor and acceptor in the double helical structure. Thus, PCT publicationWO 95/15971 and the present invention are directed to the use of nucleic acids with electron transfer moieties, including electrodes, as probes for the detection of target sequences within a sample.

In one embodiment, the present invention provides for novel gene probes, which are useful in molecular biology and diagnostic medicine. In this embodiment, single stranded nucleic acids having a predetermined sequence and covalently attached electron transfer moieties, including an electrode, are synthesized. The sequence is selected based upon a known target sequence, such that if hybridization to a complementary target sequence occurs in the region between the electron donor and the electron acceptor, electron transfer proceeds at an appreciable and detectable rate. Thus, the invention has broad general use, as a new form of labelled gene probe. In addition, the probes of the

Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker et al., Bioorganic & Medicinal Chem. Lett. 4:395 (1994); Jeffs et al., J. Biomolecular NMR 34:17 (1994); Tetrahedron Lett. 37:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins et al., Chem. Soc. Rev. (1995) pp169-176). Several nucleic acid analogs are described in Rawls, C & E News June 2, 1997 page 35. All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of electron transfer moieties, or to increase the stability and half-life of such molecules in physiological environments.

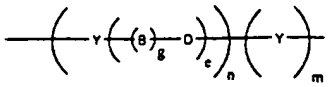
As will be appreciated by those in the art, all of these nucleic acid analogs may find use in the present invention. In addition, mixtures of naturally occurring nucleic acids and analogs can be made; for example, at the site of conductive oligomer or electron transfer moiety attachment, an analog structure may be used. Alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occuring nucleic acids and analogs may be made.

Particularly preferred are peptide nucleic acids (PNA) which includes peptide nucleic acid analogs. These backbones are substantially non-ionic under neutral conditions, in contrast to the highly charged phosphodiester backbone of naturally occurring nucleic acids. This results in two advantages. First, the PNA backbone exhibits improved hybridization kinetics. PNAs have larger changes in the melting temperature (Tm) for mismatched versus perfectly matched basepairs. DNA and RNA typically exhibit a 2-4°C drop in Tm for an internal mismatch. With the non-ionic PNA backbone, the drop is closer to 7-9°C. This allows for better detection of mismatches. Similarly, due to their non-ionic nature, hybridization of the bases attached to these backbones is relatively insensitive to salt concentration. This is particularly advantageous in the systems of the present invention, as a reduced salt hybridization solution has a lower Faradaic current than a physiological salt solution (in the range of 150 mM).

The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid contains any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xathanine hypoxathanine, isocytosine, isoguanine, etc. As used herein, the term "nucleoside" includes nucleotides and nucleoside and nucleotide analogs, and modified nucleosides such as amino

In a preferred embodiment, the conductive oligomer has the structure depicted in Structure 2:

Structure 2



As will be understood by those in the art, all of the structures depicted herein may have additional atoms or structures; i.e. the conductive oligomer of Structure 2 may be attached to electron transfer moieties, such as electrodes, transition metal complexes, organic electron transfer moieties, and metallocenes, and to nucleic acids, or to several of these. Unless otherwise noted, the conductive oligomers depicted herein will be attached at the left side to an electrode; that is, as depicted in Structure 2, the left "Y" is connected to the electrode as described herein and the right "Y", if present, is attached to the nucleic acid, either directly or through the use of a linker, as is described herein.

In this embodiment, Y is an aromatic group, n is an integer from 1 to 50, g is either 1 or zero, e is an integer from zero to 10, and m is zero or 1. When g is 1, B-D is a conjugated bond, preferably selected from acetylene, alkene, substituted alkene, amide, azo, -C=N- (including -N=C-, -CR=N- and -N=CR-), -Si=Si-, and -Si=C- (including -C=Si-, -Si=CR- and -CR=Si-). When g is zero, e is preferably 1, D is preferably carbonyl, or a heteroatom moiety, wherein the heteroatom is selected from oxygen, sulfur, nitrogen, silicon or phosphorus. Thus, suitable heteroatom moieties include, but are not limited to, -NH and -NR, wherein R is as defined herein; substituted sulfur; sulfonyl ($-SO_2-$) sulfoxide (-SO-); phosphine oxide (-PO- and -RPO-); and thiophosphine (-PS- and -RPS-). However, when the conductive oligomer is to be attached to a gold electrode, as outlined below, sulfur derivatives are not preferred.

By "aromatic group" or grammatical equivalents herein is meant an aromatic monocyclic or polycyclic hydrocarbon moiety generally containing 5 to 14 carbon atoms (although larger polycyclic rings structures may be made) and any carbocylic ketone or thioketone derivative thereof, wherein the carbon atom with the free valence is a member of an aromatic ring. Aromatic groups include arylene groups and aromatic groups with more than two atoms removed. For the purposes of this application aromatic includes heterocycle. "Heterocycle" or "heteroaryl" means an aromatic group wherein 1 to 5 of the indicated carbon atoms are replaced by a heteroatom chosen from nitrogen, oxygen, sulfur, phosphorus, boron and silicon wherein the atom with the free valence is a member of an aromatic ring, and any heterocyclic ketone and thioketone derivative thereof. Thus, heterocycle includes thienyl, furyl, pyrrolyl, pyrimidinyl, oxalyl, indolyl, purinyl, quinolyl, isoquinolyl, thiazolyl, imidozyl, etc.

hydrogen when the position is unsubstituted. It should be noted that some positions may allow two substitution groups, R and R', in which case the R and R' groups may be either the same or different.

By "alkyl group" or grammatical equivalents herein is meant a straight or branched chain alkyl group, with straight chain alkyl groups being preferred. If branched, it may be branched at one or more positions, and unless specified, at any position. The alkyl group may range from about 1 to about 30 carbon atoms (C1 -C30), with a preferred embodiment utilizing from about 1 to about 20 carbon atoms (C1 -C20), with about C1 through about C12 to about C15 being preferred, and C1 to C5 being particularly preferred, although in some embodiments the alkyl group may be much larger. Also included within the definition of an alkyl group are cycloalkyl groups such as C5 and C6 rings, and heterocyclic rings with nitrogen, oxygen, sulfur or phosphorus. Alkyl also includes heteroalkyl, with heteroatoms of sulfur, oxygen, nitrogen, and silicone being preferred. Alkyl includes substituted alkyl groups. By "substituted alkyl group" herein is meant an alkyl group further comprising one or more substitution moieties "R", as defined above.

By "amino groups" or grammatical equivalents herein is meant $-NH_2$, -NHR and $-NR_2$ groups, with R being as defined herein.

By "nitro group" herein is meant an $-NO_2$ group.

By "sulfur containing moieties" herein is meant compounds containing sulfur atoms, including but not limited to, thia-, thio- and sulfo- compounds, thiols (-SH and -SR), and sulfides (-RSR-). By "phosphorus containing moieties" herein is meant compounds containing phosphorus, including, but not limited to, phosphines and phosphates. By "silicon containing moieties" herein is meant compounds containing silicon.

By "ether" herein is meant an -O-R group. Preferred ethers include alkoxy groups, with $-O-(CH_2)_2CH_3$ and $-O-(CH_2)_4CH_3$ being preferred.

By "ester" herein is meant a -COOR group.

By "halogen" herein is meant bromine, iodine, chlorine, or fluorine. Preferred substituted alkyls are partially or fully halogenated alkyls such as CF_3, etc.

By "aldehyde" herein is meant -RCOH groups.

B-D bond may be an amide bond, and the rest of the B-D bonds may be acetylene bonds. Generally, when amide bonds are present, as few amide bonds as possible are preferable, but in some embodiments all the B-D bonds are amide bonds. Thus, as outlined above for the Y rings, one type of B-D bond may be present in the conductive oligomer within a monolayer as described below, and another type above the monolayer level, to give greater flexibility for nucleic acid hybridization.

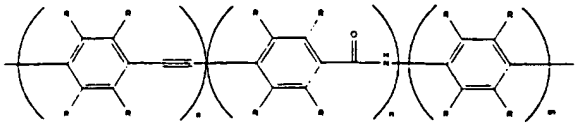
In the structures depicted herein, n is an integer from 1 to 50, although longer oligomers may also be used (see for example Schumm et al., Angew. Chem. Int. Ed. Engl. 1994 33(13):1360). Without being bound by theory, it appears that for efficient hybridization of nucleic acids on a surface, the hybridization should occur at a distance from the surface, i.e. the kinetics of hybridization increase as a function of the distance from the surface, particularly for long oligonucleotides of 200 to 300 basepairs. Accordingly, the length of the conductive oligomer is such that the closest nucleotide of the nucleic acid is positioned from about 6Å to about 100Å (although distances of up to 500Å may be used) from the electrode surface, with from about 15Å to about 60Å being preferred and from about 25Å to about 60Å also being preferred. Accordingly, n will depend on the size of the aromatic group, but generally will be from about 1 to about 20, with from about 2 to about 15 being preferred and from about 3 to about 10 being especially preferred.

In the structures depicted herein, m is either 0 or 1. That is, when m is 0, the conductive oligomer may terminate in the B-D bond or D moiety, i.e. the D atom is attached to the nucleic acid either directly or via a linker. In some embodiments, for example when the conductive oligomer is attached to a phosphate of the ribose-phosphate backbone of a nucleic acid, there may be additional atoms, such as a linker, attached between the conductive oligomer and the nucleic acid. Additionally, as outlined below, the D atom may be the nitrogen atom of the amino-modified ribose. Alternatively, when m is 1, the conductive oligomer may terminate in Y, an aromatic group, i.e. the aromatic group is attached to the nucleic acid or linker.

As will be appreciated by those in the art, a large number of possible conductive oligomers may be utilized. These include conductive oligomers falling within the Structure 2 and Structure 9 formulas, as well as other conductive oligomers, as are generally known in the art, including for example, compounds comprising fused aromatic rings or Teflon®-like oligomers, such as $-(CF_2)_n-$, $-(CHF)_n-$ and $-(CFR)_n-$. See for example, Schumm et al., angew. Chem. Intl. Ed. Engl. 33:1361 (1994);Grosshenny et al., Platinum Metals Rev. 40(1):26-35 (1996); Tour, Chem. Rev. 96:537-553 (1996); Hsung et al., Organometallics 14:4808-4815 (1995; and references cited therein, all of which are expressly incorporated by reference.

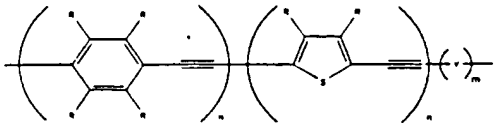
When the B-D bond is an amide bond, as in Structure 6, the conductive oligomers are pseudopeptide oligomers. Although the amide bond in Structure 6 is depicted with the carbonyl to the left, i.e. -CONH-, the reverse may also be used, i.e. -NHCO-. Particularly preferred embodiments of Structure 6 include: n is two, m is one, and R is hydrogen; n is three, m is zero, and R is hydrogen (in this embodiment, the terminal nitrogen (the D atom) may be the nitrogen of the amino-modified ribose); and the use of R groups to increase solubility.

Structure 7



Preferred embodiments of Structure 7 include the first n is two, second n is one, m is zero, and all R groups are hydrogen, or the use of R groups to increase solubility.

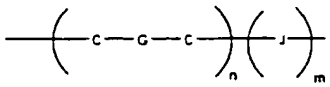
Structure 8



Preferred embodiments of Structure 8 include: the first n is three, the second n is from 1-3, with m being either 0 or 1, and the use of R groups to increase solubility.

In a preferred embodiment, the conductive oligomer has the structure depicted in Structure 9:

Structure 9



In this embodiment, C are carbon atoms, n is an integer from 1 to 50, m is 0 or 1, J is a heteroatom selected from the group consisting of oxygen, nitrogen, silicon, phosphorus, sulfur, carbonyl or sulfoxide, and G is a bond selected from alkane, alkene or acetylene, such that together with the two carbon atoms the C-G-C group is an alkene (-CH=CH-), substituted alkene (-CR=CR-) or mixtures thereof (-CH=CR- or -CR=CH-), acetylene (-C≡C-), or alkane ($-CR_2-CR_2-$, with R being either hydrogen or a substitution group as described herein). The G bond of each subunit may be the same or different than the G bonds of other subunits; that is, alternating oligomers of alkene and acetylene bonds could be used, etc. However, when G is an alkane bond, the number of alkane bonds in the oligomer should be kept to a minimum, with about six or less sigma bonds per conductive oligomer being preferred. Alkene bonds are preferred, and are generally depicted herein, although alkane and

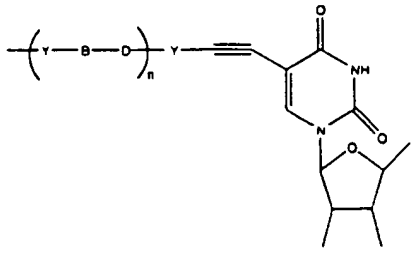
The covalent attachment of the nucleic acid and the conductive oligomer may be accomplished in several ways. In a preferred embodiment, the attachment is via attachment to the base of the nucleoside, via attachment to the backbone of the nucleic acid (either the ribose, the phosphate, or to an analogous group of a nucleic acid analog backbone), or via a transition metal ligand, as described below. The techniques outlined below are generally described for naturally occuring nucleic acids, although as will be appreciated by those in the art, similar techniques may be used with nucleic acid analogs.

In a preferred embodiment, the conductive oligomer is attached to the base of a nucleoside of the nucleic acid. This may be done in several ways, depending on the oligomer, as is described below. In one embodiment, the oligomer is attached to a terminal nucleoside, i.e. either the 3' or 5' nucleoside of the nucleic acid. Alternatively, the conductive oligomer is attached to an internal nucleoside.

The point of attachment to the base will vary with the base. While attachment at any position is possible, it is preferred to attach at positions not involved in hydrogen bonding to the complementary base. Thus, for example, generally attachment is to the 5 or 6 position of pyrimidines such as uridine, cytosine and thymine. For purines such as adenine and guanine, the linkage is preferably via the 8 position. Attachment to non-standard bases is preferably done at the comparable positions.

In one embodiment, the attachment is direct; that is, there are no intervening atoms between the conductive oligomer and the base. In this embodiment, for example, conductive oligomers with terminal acetylene bonds are attached directly to the base. Structure 11 is an example of this linkage, using a Structure 4 conductive oligomer and uridine as the base, although other bases and conductive oligomers can be used as will be appreciated by those in the art:

Structure 11



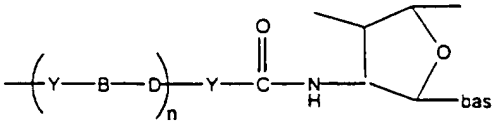
It should be noted that the pentose structures depicted herein may have hydrogen, hydroxy, phosphates or other groups such as amino groups attached. In addition, the pentose and nucleoside structures depicted herein are depicted non-conventionally, as mirror images of the normal rendering.

In a preferred embodiment, the conductive oligomer is attached to the ribose of the ribose-phosphate backbone. This may be done in several ways. As is known in the art, nucleosides that are modified at either the 2' or 3' position of the ribose with amino groups, sulfur groups, silicone groups, phosphorus groups, or oxo groups can be made (Imazawa et al., J. Org. Chem., 44:2039 (1979); Hobbs et al., J. Org. Chem. 42(4):714 (1977); Verheyden et al., J. Orrg. Chem. 36(2):250 (1971); McGee et al., J. Org. Chem. 61:781-785 (1996); Mikhailopulo et al., Liebigs. Ann. Chem. 513-519 (1993); McGee et al., Nucleosides & Nucleotides 14(6):1329 (1995), all of which are incorporated by reference). These modified nucleosides are then used to add the conductive oligomers.

A preferred embodiment utilizes amino-modified nucleosides. These amino-modified riboses can then be used to form either amide or amine linkages to the conductive oligomers. In a preferred embodiment, the amino group is attached directly to the ribose, although as will be appreciated by those in the art, short linkers such as those described herein for "Z" may be present between the amino group and the ribose.

In a preferred embodiment, an amide linkage is used for attachment to the ribose. Preferably, if the conductive oligomer of Structures 2-4 is used, m is zero and thus the conductive oligomer terminates in the amide bond. In this embodiment, the nitrogen of the amino group of the amino-modified ribose is the "D" atom of the conductive oligomer. Thus, a preferred attachment of this embodiment is depicted in Structure 13 (using the Structure 4 conductive oligomer):

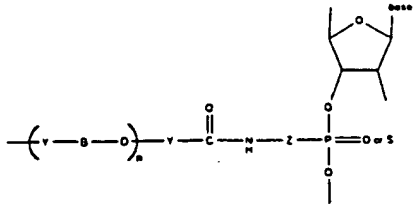
Structure 13



As will be appreciated by those in the art, Structure 13 has the terminal bond fixed as an amide bond.

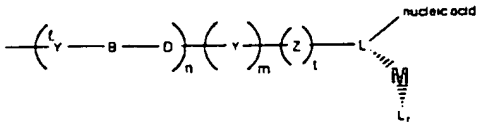
In a preferred embodiment, a heteroatom linkage is used, i.e. oxo, amine, sulfur, etc. A preferred embodiment utilizes an amine linkage. Again, as outlined above for the amide linkages, for amine linkages, the nitrogen of the amino-modified ribose may be the "D" atom of the conductive oligomer when the Structure 4 conductive oligomer is used. Thus, for example, Structures 14 and 15 depict nucleosides with the Structures 4 and 10 conductive oligomers, respectively, using the nitrogen as the heteroatom, athough other heteroatoms can be used:

Structure 17

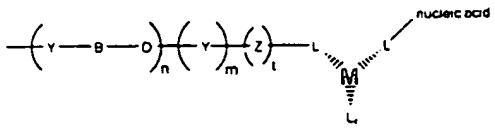


In a preferred embodiment, the conductive oligomer is covalently attached to the nucleic acid via a transition metal ligand. In this embodiment, the conductive oligomer is covalently attached to a ligand which provides one or more of the coordination atoms for a transition metal. In one embodiment, the ligand to which the conductive oligomer is attached also has the nucleic acid attached, as is generally depicted below in Structure 18. Alternatively, the conductive oligomer is attached to one ligand, and the nucleic acid is attached to another ligand, as is generally depicted below in Structure 19. Thus, in the presence of the transition metal, the conductive oligomer is covalently attached to the nucleic acid. Both of these structures depict Structure 4 conductive oligomers, although other oligomers may be utilized. Structures 18 and 19 depict two representative structures:

Structure 18



Structure 19



In the structures depicted herein, M is a metal atom, with transition metals being preferred. Suitable transition metals for use in the invention include, but are not limited to, cadmium (Cd), copper (Cu), cobalt (Co), palladium (Pd), zinc (Zn), iron (Fe), ruthenium (Ru), rhodium (Rh), osmium (Os), rhenium (Re), platinium (Pt), scandium (Sc), titanium (Ti), Vanadium (V), chromium (Cr), manganese (Mn), nickel (Ni), Molybdenum (Mo), technetium (Tc), tungsten (W), and iridium (Ir). That is, the first series of transition metals, the platinum metals (Ru, Rh, Pd, Os, Ir and Pt), along with Fe, Re, W, Mo and Tc, are preferred. Particularly preferred are ruthenium, rhenium, osmium, platinium, cobalt and iron.

L are the co-ligands, that provide the coordination atoms for the binding of the metal ion. As will b appr ciated by those in the art, th number and nature of the co-ligands will depend on the

The oxygen, sulfur, phosphorus and nitrogen-donating ligands are attached in such a manner as to allow the heteroatoms to serve as coordination atoms.

In a preferred embodiment, organometallic ligands are used. In addition to purely organic compounds for use as redox moieties, and various transition metal coordination complexes with δ-bonded organic ligand with donor atoms as heterocyclic or exocyclic substituents, there is available a wide variety of transition metal organometallic compounds with π-bonded organic ligands (see Advanced Inorganic Chemistry, 5th Ed., Cotton & Wilkinson, John Wiley & Sons, 1988, chapter 26; Organometallics, A Concise Introduction, Elschenbroich et al., 2nd Ed., 1992, VCH; and Comprehensive Organometallic Chemistry II, A Review of the Literature 1982-1994, Abel et al. Ed., Vol. 7, chapters 7, 8, 10 & 11, Pergamon Press, hereby expressly incorporated by reference). Such organometallic ligands include cyclic aromatic compounds such as the cyclopentadienide ion [$C_5H_5(-1)$] and various ring substituted and ring fused derivatives, such as the indenylide (-1) ion, that yield a class of bis(cyclopentadieyl)metal compounds, (i.e. the metallocenes); see for example Robins et al., J. Am. Chem. Soc. 104:1882-1893 (1982); and Gassman et al., J. Am. Chem. Soc. 108:4228-4229 (1986), incorporated by reference. Of these, ferrocene [$(C_5H_5)_2Fe$] and its derivatives are prototypical examples which have been used in a wide variety of chemical (Connelly et al., Chem. Rev. 96:877-910 (1996), incorporated by reference) and electrochemical (Geiger et al., Advances in Organometallic Chemistry 23:1-93; and Geiger et al., Advances in Organometallic Chemistry 24:87, incorporated by reference) electron transfer or "redox" reactions. Metallocene derivatives of a variety of the first, second and third row transition metals are potential candidates as redox moieties that are covalently attached to either the ribose ring or the nucleoside base of nucleic acid. Other potentially suitable organometallic ligands include cyclic arenes such as benzene, to yield bis(arene)metal compounds and their ring substituted and ring fused derivatives, of which bis(benzene)chromium is a prototypical example. Other acyclic π-bonded ligands such as the allyl(-1) ion, or butadiene yield potentially suitable organometallic compounds, and all such ligands, in conjuction with other π-bonded and δ-bonded ligands constitute the general class of organometallic compounds in which there is a metal to carbon bond. Electrochemical studies of various dimers and oligomers of such compounds with bridging organic ligands, and additional non-bridging ligands, as well as with and without metal-metal bonds are potential candidate redox moieties in nucleic acid analysis.

When one or more of the co-ligands is an organometallic ligand, the ligand is generally attached via one of the carbon atoms of the organometallic ligand, although attachment may be via other atoms for heterocyclic ligands. Preferred organometallic ligands include metallocene ligands, including substituted derivatives and the metalloceneophanes (see page 1174 of Cotton and Wilkenson, supra) For example, derivatives of metallocene ligands such as methylcyclopentadienyl, with multiple methyl

ligand may contain a multiplicity of amino groups so as to form a polydentate ligand which binds the metal ion. Other preferred ligands include cyclopentadiene and phenanthroline.

As described herein, the compositions described herein of nucleosides covalently attached to conductive oligomers may be incorporated into a longer nucleic acid at any number of positions, including either the 5' or 3' terminus of the nucleic acid or any internal position. As is outlined below, this is generally done by adding a nucleotide with a covalently attached conductive oligomer to an oligonucleotide synthetic reaction at any position. After synthesis is complete, the nucleic acid with the covalently attached conductive oligomer is attached to an electrode. Thus, any number of additional nucleotides, modified or not, may be included at any position. Alternatively, the compositions are made via post-nucleic acid synthesis modifications.

The total length of the nucleic acid will depend on its use. Generally, the nucleic acid compositions of the invention are useful as oligonucleotide probes. As is appreciated by those in the art, the length of the probe will vary with the length of the target sequence and the hybridization and wash conditions. Generally, oligonucleotide probes range from about 8 to about 50 nucleotides, with from about 10 to about 30 being preferred and from about 12 to about 25 being especially preferred. In some cases, very long probes may be used, e.g. 50 to 200-300 nucleotides in length.

Also of consideration is the distance between the nucleoside containing the electrode, i.e. a first electron transfer moiety, and the nucleoside containing a second electron transfer moiety. Electron transfer proceeds between the two electron transfer moieties. Since the rate of electron transfer is distance dependent, the distance between the two electron transfer moieties preferably ranges from about 1 to about 30 basepairs, with from about 1 to about 20 basepairs being preferred and from about 2 to about 10 basepairs being particularly preferred and from about 2 to 6 being especially preferred. However, probe specificity can be increased by adding oligonucleotides on either side of the electron transfer moieties, thus increasing probe specificity without increasing the distance an electron must travel.

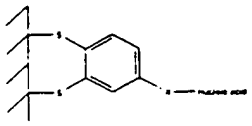
Thus, in the structures depicted herein, nucleosides may be replaced with nucleic acids.

In a preferred embodiment, the conductive oligomers with covalently attached nucleosides or nucleic acids as depicted herein are covalently attached to an electrode. Thus, one end or terminus of the conductive oligomer is attached to th nucleoside or nucleic acid, and the other is attached to an electrode. In some embodiments it may b desirable to have the conductive oligomer attached at a position other than a t rminus, or ev n to have a branched conductive oligomer that is attached to an

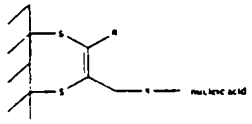
carbon based electrodes are used, A may be an amino moiety (preferably a primary amine; see for example Deinhammer et al., Langmuir 10:1306-1313 (1994)). Thus, preferred A moieties include, but are not limited to, silane moieties, sulfur moieties (including alkyl sulfur moieties), and amino moieties. In a preferred embodiment, epoxide type linkages with redox polymers such as are known in the art are not used.

Although depicted herein as a single moiety, the conductive oligomer may be attached to the electrode with more than one "A" moiety; the "A" moieties may be the same or different. Thus, for example, when the electrode is a gold electrode, and "A" is a sulfur atom or moiety, such as generally depicted below in Structure 27, multiple sulfur atoms may be used to attach the conductive oligomer to the electrode, such as is generally depicted below in Structures 24, 25 and 26. As will be appreciated by those in the art, other such structures can be made. In Structures 24, 25 and 26, the A moiety is just a sulfur atom, but substituted sulfur moieties may also be used.

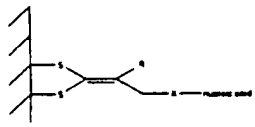
Structure 24



Structure 25



Structure 26



It should also be noted that similar to Structure 26, it may be possible to have a a conductive oligomer terminating in a single carbon atom with three sulfur moities attached to the electrode.

In a preferred embodiment, the electrode is a gold electrode, and attachment is via a sulfur linkage as is well known in the art, i.e. the A moiety is a sulfur atom or moiety. Although the exact characteristics of the gold-sulfur attachment are not known, this linkage is considered covalent for the purposes of this invention. A representative structure is depicted in Structure 27. Structure 27 depicts the "A"

In this embodiment, the number of different probe species of oligonucleotides may vary widely, from one to thousands, with from about 4 to about 100,000 being preferred, and from about 10 to about 10,000 being particularly preferred.

In a preferred embodiment, the electrode further comprises a passivation agent, preferably in the form of a monolayer on the electrode surface. As outlined above, the efficiency of oligonucleotide hybridization may increase when the oligonucleotide is at a distance from the electrode. A passivation agent layer facilitates the maintenance of the nucleic acid away from the electrode surface. In addition, a passivation agent serves to keep charge carriers away from the surface of the electrode. Thus, this layer helps to prevent electrical contact between the electrodes and the electron transfer moieties, or between the electrode and charged species within the solvent. Such contact can result in a direct "short circuit" or an indirect short circuit via charged species which may be present in the sample. Accordingly, the monolayer of passivation agents is preferably tightly packed in a uniform layer on the electrode surface, such that a minimum of "holes" exist. Alternatively, the passivation agent may not be in the form of a monolayer, but may be present to help the packing of the conductive oligomers or other characteristics.

The passivation agents thus serve as a physical barrier to block solvent accesibility to the electrode. As such, the passivation agents themselves may in fact be either (1) conducting or (2) nonconducting, i.e. insulating, molecules. Thus, in one embodiment, the passivation agents are conductive oligomers, as described herein, with or without a terminal group to block or decrease the transfer of charge to the electrode. Other passivation agents which may be conductive include oligomers of $-(CF_2)_n-$, $-(CHF)_n-$ and $-(CFR)_n-$. In a preferred embodiment, the passivation agents are insulator moieties.

An "insulator" is a substantially nonconducting oligomer, preferably linear. By "substantially nonconducting" herein is meant that the rate of electron transfer through the insulator is slower than the rate of electron transfer through the stacked π-orbitals of double stranded nucleic acid. Stated differently, the electrical resistance of the insulator is higher than the electrical resistance of the nucleic acid. In a preferred embodiment, the rate of electron transfer through the insulator is slower than or comparable to the rate through single stranded nucleic acid. Similarly, the rate of electron transfer through the insulator is preferrably slower than the rate through the conductive oligomers described herein. It should be noted however, as outlined in the Examples, that even oligomers generally considered to be insulators, such as $-(CH_2)_{16}$ molecules, still may transfer electrons, albeit at a slow rate.

The passivation agents are generally attached to the electrode in the same manner as the conductive oligomer, and may use the same "A" linker as defined above.

It has been found that the present compositions result in excellent hybridization kinetics of target sequence hybridizing to probes attached to a surface. Thus, the compositions and methods of the present invention may also be used in nucleic acid detection systems that do not rely on electron transfer for detection.

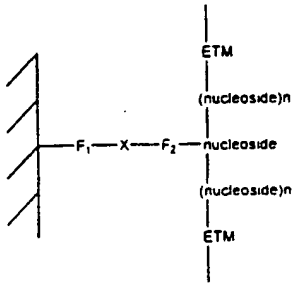
Accordingly, in a preferred embodiment, the compositions of the present invention find use in standard nucleic acid assays, such as general array-type technologies, i.e. the electrode may serve just as a solid support, with detection proceeding using techniques well known in the art, such as fluoroscence or radioisotope labelling. In this embodiment, the compositions may comprise a conductive oligomer covalently attached to a nucleoside or nucleic acid. It will be recognized by those in the art that the conductive oligomers in this embodiment may not be functioning as conductive oligomers but rather as linkers that can be used to keep the nucleic acids off the surface. The conductive oligomer, or linker, in this case may have the structure depicted in Structures 2, 3, 4, 9 or 10. However, when the linker has the structure depicted in Structure 9, preferably at least one of the G bonds is not alkane, particularly when m=0.

In a preferred embodiment, the composition comprises (a) a solid support comprising a monolayer of passivation agent; (b) a nucleic acid comprising at least one nucleoside, wherein the nucleic acid is covalently attached to the solid support with a linker. The solid support is the electrode, which is not necessarily functioning as an electron transfer moiety in this embodiment. The monolayer of passivation agent is shown herein to result in excellent hybridization kinetics and can therefore be quite useful in both electron-transfer based and traditional nucleic acid detection schemes. The linkers are preferably the conductive oligomers of the invention, although as outlined above, they may not be functioning as conductive moieties. In this embodiment, the conductive oligomer, or linker, in this case, may have the structure depicted in Structures 2, 3, 4, 9 or 10. However, when the linker has the structure depicted in Structure 9, preferably at least one of the G bonds is not alkane, particularly when m=0.

In this embodiment, it is possible to have each nucleic acid be the same, as an "anchor sequence", such that a second sequence can be added which contains the probe sequence and a sequence complementary to the anchor sequence. In this way, standard arrays of using either the same or different anchor sequences can be made, which then can be used to generate custom arrays using novel probe sequences linked to complementary anchor regions.

uniform signal. Alternatively, the additional electron transfer moieties may be different and/or placed at different distances from the conductive oligomer.

Structure 29A



The terms "electron donor moiety", "electron acceptor moiety", and "electron transfer moieties" or grammatical equivalents herein refers to molecules capable of electron transfer under certain conditions. It is to be understood that electron donor and acceptor capabilities are relative; that is, a molecule which can lose an electron under certain experimental conditions will be able to accept an electron under different experimental conditions. It is to be understood that the number of possible electron donor moieties and electron acceptor moieties is very large, and that one skilled in the art of electron transfer compounds will be able to utilize a number of compounds in the present invention. Preferred electron transfer moieties include, but are not limited to, transition metal complexes, organic electron transfer moieties, and electrodes.

In a preferred embodiment, the electron transfer moieties are transition metal complexes. Transition metals are those whose atoms have a partial or complete d shell of electrons. Suitable transition metals for use in the invention are listed above.

The transition metals are complexed with a variety of ligands, L, defined above, to form suitable transition metal complexes, as is well known in the art.

In addition to transition metal complexes, other organic electron donors and acceptors may be covalently attached to the nucleic acid for use in the invention. These organic molecules include, but are not limited to, riboflavin, xanthene dyes, azine dyes, acridine orange, *N,N'*-dimethyl-2,7-diazapyrenium dichloride (DAP^{2+}), methylviologen, ethidium bromide, quinones such as N,N'-dimethylanthra(2,1,9-*def*:6,5,10-*d'e'f'*)diisoquinoline dichloride ($ADIQ^{2+}$); porphyrins ([meso-tetrakis(N-methyl-x-pyridinium)porphyrin tetrachlorid], varlamine blue B hydrochloride, Bindschedler's green; 2,6-dichloroindophenol, 2,6-dibromophenolindophenol; Brilliant crest blue (3-amino-9-dimethyl-amino-10-methylphenoxyazine chloride), methylene blue; Nile blue A (aminoaphthodiethylaminophenoxazine

In a preferred embodiment, the second electron transfer moiety is attached to the base of a nucleoside, as is generally outlined above for attachment of the conductive oligomer. This is preferably done to the base of an internal nucleoside. Surprisingly and unexpectedly, this attachment does not perturb the Watson-Crick basepairing of the base to which the electron transfer moiety is attached, as long as the moiety is not too large. In fact, it appears that attachment at this site actually results in less perturbation than attachment at the ribose of the ribose-phosphate backbone, as measured by nucleic acid melting curves.

Thus, when attachment to an internal base is done, the size of the second electron transfer moiety should be such that the structure of double stranded nucleic acid containing the base-attached electron transfer moiety is not significantly disrupted, and will not disrupt the annealing of single stranded nucleic acids. Preferrably, then, ligands and full second electron transfer moieties are generally smaller than the size of the major groove of double stranded nucleic acid.

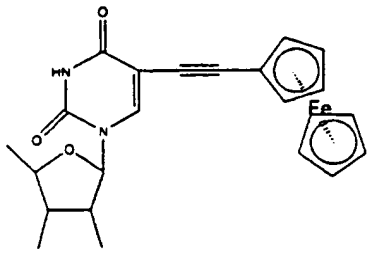
Alternatively, the second electron transfer moiety can be attached to the base of a terminal nucleoside Thus, when the target sequence to be detected is n nucleosides long, a probe can be made which has the second electron transfer moiety attached at the n base. Alternatively, the probe may contain an extra terminal nucleoside at an end of the nucleic acid (n + 1 or n + 2), which are used to covalently attach the electron transfer moieties but which do not participate in basepair hybridization. Additionally, it is preferred that upon probe hybridization, the terminal nucleoside containing the electron transfer moiety covalently attached at the base be directly adjacent to Watson-Crick basepaired nucleosides; that is, the electron transfer moiety should be as close as possible to the stacked π-orbitals of the bases such that an electron travels through a minimum of σ bonds to reach the "π-way", or alternatively can otherwise electronically contact the π-way.

The covalent attachment to the base will depend in part on the second electron transfer moiety chosen, but in general is similar to the attachment of conductive oligomers to bases, as outlined above. In a preferred embodiment, the second electron transfer moiety is a transition metal complex, and thus attachment of a suitable metal ligand to the base leads to the covalent attachment of the electron transfer moiety. Alternatively, similar types of linkages may be used for the attachment of organic electron transfer moieties, as will be appreciated by those in the art.

In one embodiment, the C4 attached amino group of cytosine, the C6 attached amino group of adenine, or the C2 attached amino group of guanine may be used as a transition metal ligand, although in this embodiment attachment at a terminal base is preferred since attachment at these positions will perturb Watson-Crick basepairing.

In a preferred embodiment, the second electron transfer moiety attached to a nucleoside is a metallocene; i.e. the L and L_r of Structure 32 are both metallocene ligands, L_m, as described above. Structure 33 depicts a preferred embodiment wherein the metallocene is ferrocene, and the base is uridine, although other bases may be used:

Structure 33



Preliminary data suggest that Structure 33 may cyclize, with the second acetylene carbon atom attacking the carbonyl oxygen, forming a furan-like structure.

Preferred metallocenes include ferrocene, cobaltocene and osmiumocene.

Thus, in a preferred embodiment, the invention provides metallocenes covalently attached to nucleosides. In a preferred embodiment, the metallocene is attached to the base of a nucleoside. In alternate preferred embodiment, the metallocene is attached to the ribose of a nucleoside. Alternatively, the metallocene may be attached to the phosphate of the backbone, although this is generally not preferred. If attachment is to the phosphate, generally there will be no more than about 2-4 atoms between the phosphate atom and a carbon of a ring of the metallocene. In a preferred embodiment, the metallocene is ferrocene or substituted ferrocene.

In a preferred embodiment, the second electron transfer moiety is attached to a ribose at any position of the ribose-phosphate backbone of the nucleic acid, i.e. either the 5' or 3' terminus or any internal nucleoside. As is known in the art, nucleosides that are modified at either the 2' or 3' position of the ribose can be made, with nitrogen, oxygen, sulfur and phosphorus-containing modifications possible. Amino-modified ribose is preferred. See generally PCT publication WO 95/15971, incorporated herein by reference. These modification groups may be used as a transition metal ligand, or as a chemically functional moiety for attachment of other transition metal ligands and organometallic ligands, or organic electron donor moieties as will be appreciated by those in the art. In this embodiment, a linker such as depicted herein for "Z" may be used as well, or a conductive oligomer between the ribose and the electron transfer moiety. Preferred embodiments utilize attachment at the 2' or 3' position of the ribose, with the 2' position being preferred. Thus for xample, the conductive oligomers depicted in

transfer moiety covalently attached. Any combination of positions of electron transfer moiety attachment can be made; i.e. an electrode at the 5' terminus, a second electron transfer moiety at an internal position; electrode at the 5' terminus, second moiety at the 3' end; second moiety at the 5' terminus, electrode at an internal position; both electrode and second moiety at internal positions; electrode at an internal position, second moiety at the 3' terminus, etc. A preferred embodiment utilizes both the electrode and the second electron transfer moiety attached to internal nucleosides.

The compositions of the invention may additionally contain one or more labels at any position. By "label" herein is meant an element (e.g. an isotope) or chemical compound that is attached to enable the detection of the compound. Preferred labels are radioactive isotopic labels, and colored or fluorescent dyes. The labels may be incorporated into the compound at any position. In addition, the compositions of the invention may also contain other moieties such as cross-linking agents to facilitate cross-linking of the target-probe complex. See for example, Lukhtanov et al., Nucl. Acids. Res. 24(4):683 (1996) and Tabone et al., Biochem. 33:375 (1994), both of which are expressly incorporated by reference.

The compositions of the invention are generally synthesized as outlined below, generally utilizing techniques well known in the art. As will be appreciated by those in the art, many of the techniques outlined below are directed to nucleic acids containing a ribose-phosphate backbone. However, as outlined above, many alternate nucleic acid analogs may be utilized, some of which may not contain either ribose or phosphate in the backbone. In these embodiments, for attachment at positions other than the base, attachment is done as will be appreciated by those in the art, depending on the backbone. Thus, for example, attachment can be made at the carbon atoms of the PNA backbone, as is described below, or at either terminus of the PNA.

The compositions may be made in several ways. A preferred method first synthesizes a conductive oligomer attached to the nucleoside, with addition of additional nucleosides followed by attachment to the electrode. A second electron transfer moiety, if present, may be added prior to attachment to the electrode or after. Alternatively, the whole nucleic acid may be made and then the completed conductive oligomer added, followed by attachment to the electrode. Alternatively, the conductive oligomer and monolayer (if present) are attached to the electrode first, followed by attachment of the nucleic acid. The latter two methods may be preferred when conductive oligomers are used which are not stable in the solvents and under the conditions used in traditional nucleic acid synthesis.

In a preferred embodiment, the compositions of the invention are made by first forming the conductive oligomer covalently attached to the nucleoside, followed by the addition of additional nucleosides to

addition, the palladium cross-coupling reactions may be altered to prevent dimerization problems; i.e. two conductive oligomers dimerizing, rather than coupling to the base.

Alternatively, attachment to the base may be done by making the nucleoside with one unit of the oligomer, followed by the addition of others.

Once the modified nucleosides are prepared, protected and activated, prior to attachment to the electrode, they may be incorporated into a growing oligonucleotide by standard synthetic techniques (Gait, Oligonucleotide Synthesis: A Practical Approach, IRL Press, Oxford, UK 1984; Eckstein) in several ways. In one embodiment, one or more modified nucleosides are converted to the triphosphate form and incorporated into a growing oligonucleotide chain by using standard molecular biology techniques such as with the use of the enzyme DNA polymerase I, T4 DNA polymerase, T7 DNA polymerase, Taq DNA polymerase, reverse transcriptase, and RNA polymerases. For the incorporation of a 3' modified nucleoside to a nucleic acid, terminal deoxynucleotidyltransferase may be used. (Ratliff, Terminal deoxynucleotidyltransferase. In The Enzymes, Vol 14A. P.D. Boyer ed. pp 105-118. Academic Press, San Diego, CA. 1981). Alternatively, and preferably, the amino nucleoside is converted to the phosphoramidite or H-phosphonate form, which are then used in solid-phase or solution syntheses of oligonucleotides. In this way the modified nucleoside, either for attachment at the ribose (i.e. amino- or thiol-modified nucleosides) or the base, is incorporated into the oligonucleotide at either an internal position or the 5' terminus. This is generally done in one of two ways. First, the 5' position of the ribose is protected with 4',4-dimethoxytrityl (DMT) followed by reaction with either 2-cyanoethoxy-bis-diisopropylaminophosphine in the presence of diisopropylammonium tetrazolide, or by reaction with chlorodiisopropylamino 2'-cyanoethyoxyphosphine, to give the phosphoramidite as is known in the art; although other techniques may be used as will be appreciated by those in the art. See Gait, supra; Caruthers, Science 230:281 (1985), both of which are expressly incorporated herein by reference.

For attachment of an electron transfer moiety to the 3' terminus, a preferred method utilizes the attachment of the modified nucleoside to controlled pore glass (CPG) or other oligomeric supports. In this embodiment, the modified nucleoside is protected at the 5' end with DMT, and then reacted with succinic anhydride with activation. The resulting succinyl compound is attached to CPG or other oligomeric supports as is known in the art. Further phosphoramidite nucleosides are added, either modified or not, to the 5' end after deprotection. Thus, the present invention provides conductive oligomers coval ntly attached to nucleosid s attached to solid oligomeric supports such as CPG, and phosphoramidite derivatives of the nucleosides of the invention.

36(34):6017-6020 (1995); Tzalis et al., Tetrahedron Lett. 36(2):3489-3490 (1995); and Tzalis et al., Chem. Communications (in press) 1996, all of which are hereby expressly incorporated by reference. See also the examples, which describes the synthesis of a metallocene attached via an acetylene linkage to the base.

In one embodiment, the nucleosides are made with transition metal ligands, incorporated into a nucleic acid, and then the transition metal ion and any remaining necessary ligands are added as is known in the art. In an alternative embodiment, the transition metal ion and additional ligands are added prior to incorporation into the nucleic acid.

In some embodiments, as outlined herein, conductive oligomers are used between the second electron transfer moieties and the nucleosides. These are made using the techniques described herein, with the addition of the terminal second electron transfer moiety.

Once the nucleic acids of the invention are made, with a covalently attached conductive oligomer and optionally a second electron transfer moiety, the conductive oligomer is attached to the electrode. The method will vary depending on the type of electrode used. As is described herein, the conductive oligomers are generally made with a terminal "A" linker to facilitate attachment to the electrode. For the purposes of this application, a sulfur-gold attachment is considered a covalent attachment.

In a preferred embodiment, conductive oligomers are covalently attached via sulfur linkages to the electrode. However, surprisingly, traditional protecting groups for use of attaching molecules to gold electrodes are generally ideal for use in both synthesis of the compositions described herein and inclusion in oligonucleotide synthetic reactions. Accordingly, the present invention provides novel methods for the attachment of conductive oligomers to gold electrodes, utilizing unusual protecting groups, including ethylpyridine, and trimethylsilylethyl as is depicted in the Figures.

This may be done in several ways. In a preferred embodiment, the subunit of the conductive oligomer which contains the sulfur atom for attachment to the electrode is protected with an ethyl-pyridine or trimethylsilylethyl group. For the former, this is generally done by contacting the subunit containing the sulfur atom (preferably in the form of a sulfhydryl) with a vinyl pyridine group or vinyl trimethylsilylethyl group under conditions whereby an ethylpyridine group or trimethylsilylethyl group is added to the sulfur atom.

This subunit also generally contains a functional moiety for attachment of additional subunits, and thus additional subunits are attached to form the conductive oligomer. Th conductive oligomer is then

functional moiety suitable for attachment of a completed nucleic acid; or (5) formation of a monolayer which includes conductive oligomers which terminate in a functional moiety suitable for nucleic acid synthesis, i.e. the nucleic acid is synthesized on the surface of the monolayer as is known in the art. Such suitable functional moieties include, but are not limited to, nucleosides, amino groups, carboxyl groups, protected sulfur moieties, or hydroxyl groups for phosphoramidite additions. The examples describe the formation of a monolayer on a gold electrode using the preferred method (1).

In a preferred embodiment, the nucleic acid is a peptide nucleic acid or analog. In this embodiment, the invention provides peptide nucleic acids with at least one covalently attached chemical substituent. By "chemical substituent" herein is meant any chemical or biological moiety. Preferred chemical substituents include, but are not limited to, chemical functional moieties such as amino groups, thiol groups, carbon atoms, etc., which can be used to attach other moieties; labels; signaling moieties which can be used for detection; etc. Accordingly, chemical substitutents include, but are not limited to, electron transfer moieties, including electrodes, transition metal complexes, and organic electron transfer moieties; other transition metal complexes; other labels including fluoroscent labels, radioisotope labels and chemiluminescent labels; haptens such as biotin, avidin, and digoxigenin; antigens; proteins such as antibodies, ligands, receptors, and enzymes; conductive oligomers and other polymers; and other components of binding pairs such as nucleic acids.

In a preferred embodiment, the chemical substituents are covalently attached to an monomeric subunit of the PNA. By "monomeric subunit of PNA" herein is meant the -NH-CH_2CH_2-N($COCH_2$-Base)-CH_2-CO- monomer, or derivatives (herein included within the definition of "nucleoside") of PNA. For example, the number of carbon atoms in the PNA backbone may be altered; see generally Nielsen et al., Chem. Soc. Rev. 1997 page 73, which discloses a number of PNA derivatives, herein expressly incorporated by reference. Similarly, the amide bond linking the base to the backbone may be altered; phosphoramide and sulfuramide bonds may be used.

In a preferred embodiment, the chemical substituents are attached to an internal monomeric subunit. By "internal" herein is meant that the monomeric subunit is not either the N-terminal monomeric subunit or the C-terminal monomeric subunit.

In this embodiment, the chemical substituents can be attached either to a base or to the backbone of the monomeric subunit. In a preferred embodiment, at least one chemical substituent is attached to an internal base. Attachment to the base is done as outlined herein or known in the literature. In general, the chemical substituents are added to a base which is then incorporated into a PNA as outlined herein. The bas may be either protected, as required for incorporation into the PNA synthetic

Once made, the compositions find use in a number of applications, as described herein.

In a preferred embodiment, the compositions of the invention are used as probes in hybridization assays to detect target sequences in a sample. The term "target sequence" or grammatical equivalents herein means a nucleic acid sequence on a single strand of nucleic acid. The target sequence may be a portion of a gene, a regulatory sequence, genomic DNA, cDNA, RNA including mRNA and rRNA, or others. It may be any length, with the understanding that longer sequences are more specific. As will be appreciated by those in the art, the complementary target sequence may take many forms. For example, it may be contained within a larger nucleic acid sequence, i.e. all or part of a gene or mRNA, a restriction fragment of a plasmid or genomic DNA, among others. As is outlined more fully below, probes are made to hybridize to target sequences to determine the presence or absence of the target sequence in a sample. Generally speaking, this term will be understood by those skilled in the art.

If required, the target sequence is prepared using known techniques. For example, the sample may be treated to lyse the cells, using known lysis buffers, electroporation, etc., with purification and/or amplification occuring as needed, as will be appreciated by those in the art.

The probes of the present invention are designed to be complementary to the target sequence, such that hybridization of the target sequence and the probes of the present invention occurs. As outlined below, this complementarity need not be perfect; there may be any number of base pair mismatches which will interfere with hybridization between the target sequence and the single stranded nucleic acids of the present invention. However, if the number of mutations is so great that no hybridization can occur under even the least stringent of hybridization conditions, the sequence is not a complementary target sequence.

A variety of hybridization conditions may be used in the present invention, including high, moderate and low stringency conditions; see for example Maniatis et al., Molecular Cloning: A Laboratory Manual, 2d Edition, 1989, and Short Protocols in Molecular Biology, ed. Ausubel, et al, hereby incorporated by referenece. The hybridization conditions may also vary when a non-ionic backbone, i.e. PNA is used, as is known in the art. In addition, cross-linking agents may be added after target binding to cross-link, i.e. covalently attach, the two strands of the hybridization complex.

In a preferred embodiment, single stranded nucleic acids are made which contain a first electron transfer moiety, an electrode, and at least a second electron tranfer moiety. Hybridization to a target sequence forms a double stranded hybridization compl x. In a hybridization complex, at least the

indicators serve as an electron transfer moiety that will preferentially associate with double stranded nucleic acid is added, usually reversibly, similar to the method of Millan et al., Anal. Chem. 65:2317-2323 (1993); Millan et al., Anal. Chem. 662943-2948 (1994), both of which are hereby expressly incorporated by reference. Hybridization indicators include intercalators and minor and/or major groove binding moieties. In a preferred embodiment, intercalators may be used; since intercalation generally only occurs in the presence of double stranded nucleic acid, only in the presence of target hybridization will electron transfer occur. Intercalating transition metal complex electron transfer moieties are known in the art. Similarly, major or minor groove binding moieties, such as methylene blue, may also be used in this embodiment.

In addition, hybridization indicators may be used in any or all of the other systems of the invention; for example, they may be added to facilitate, quench or amplify the signal generated by the system, in addition to the covalently attached electron transfer moieties. For example, it has been shown by Millan, above, that some hybridization indicators may preferentially bind to perfectly complementary double stranded nucleic acids over nucleic acids containing mismatches. This could serve to contribute additional information about the system. Similarly, electronic coupling could be increased due to hybridization indicator binding. Alternatively, quenching of the electron transfer signal could be acheived using hybridization indicators, whereby the electrons would flow between the second electron tranfer moiety and the hybridization indicator, rather than the electrode.

A further embodiment utilizes compositions comprising a) a first single stranded nucleic acid covalently attached to an electrode via a conductive oligomer; b) a second single stranded nucleic acid containing a second electron transfer moiety; and c) an intervening single stranded nucleic acid, which may or may not be labelled or contain an electron transfer moiety. As generally outlined in PCT WO 95/15971, the first single stranded nucleic acid hybridizes to the first target domain, the second single stranded nucleic acid hybridizes to the second target domain, and the intervening nucleic acid hybridizes to the intervening target domain, with electron transfer upon initiation. The intervening nucleic acid may be any length, taking into consideration the parameters for the distance between the electron transfer moieties, although it may be a single nucleoside.

In addition, the first and second, or first, intervening and second, nucleic acids may be ligated together prior to the electron transfer reaction, using standard molecular biology techniques such as the use of a ligase.

In one embodiment, the compositions of the invention are used to detect mismatches in a complementary target sequence. A mismatch, whether it be a substitution, insertion or deletion of a

voltages of roughly 200 mV, ferrocene is converted to ferricenium, which then transfers an electron to the nucleic acid. If this nucleic acid is double stranded, transfer proceeds rapidly through the double stranded nucleic acid, through the conductive oligomer, to the electrode. Now the ferricyanide can be oxidized to transfer an electron to the ETM. In this way, the electron source (or co-reductant) serves to amplify the signal generated in the system, as the electron source molecules rapidly and repeatedly donate electrons to the second ETM attached to the nucleic acid. The rate of electron donation or acceptance will be limited by the rate of diffusion of the co-reductant, which in turn is affected by the concentration and size, etc.

Alternatively, input electron sources that have lower redox potentials than the second ETM are used. At voltages less than the redox potential of the ETM, but higher than the redox potential of the electron source, the input source such as ferrocyanide is unable to be oxided and thus is unable to donate an electron to the ETM; i.e. no electron transfer occurs. The use of electron source molecules, however, is only possible when an insulating or passivation layer is present, since otherwise the source molecule will transfer electrons directly to the electrode. Accordingly, in a preferred embodiment, an electron source is used in solution to amplify the signal generated in the presence of hybridized target sequence.

In an alternate preferred embodiment, an input electron source is used that has a higher redox potential than the second electron transfer moiety (ETM) covalently attached to the probe nucleic acid. For example, luminol, an electron source, has a redox potential of roughly 720 mV. At voltages higher than the redox potential of the ETM, but lower than the redox potential of the electron source, i.e. 200 - 720 mV, the ferrocene is oxided, and transfers a single electron to the electrode via the conductive oligomer. However, the ETM is unable to accept any electrons from the luminol electron source, since the voltages are less than the redox potential of the luminol. However, at or above the redox potential of luminol, the luminol then transfers an electron to the ETM, allowing rapid and repeated electron transfer. In this way, the electron source (or co-reductant) serves to amplify the signal generated in the system, as the electron source molecules rapidly and repeatedly donate electrons to the second ETM attached to the nucleic acid.

Luminol has the added benefit of becoming a chemiluminiscent species upon oxidation (see Jirka et al., Analytica Chimica Acta 284:345 (1993)), thus allowing photo-detection of electron transfer through double-stranded nucleic acid. Thus, as long as the luminol is unable to contact the electrode directly, i.e. in the presence of a passivation layer, luminol can only be oxidized by transferring an electron to the second electron transfer moiety on the nucleic acid (e.g. ferrocene). When double stranded nucleic acid is not present, i.e. when the target sequenc is not hybridized to the composition of the

spectral changes upon oxidation and reduction resulting in highly sensitive monitoring of electron transfer. Such examples include $Ru(NH_3)_4py$ and $Ru(bpy)_2im$ as preferred examples. It should be understood that only the donor or acceptor that is being monitored by absorbance need have ideal spectral characteristics. That is, the electron acceptor can be optically invisible if only the electron donor is monitored for absorbance changes.

In a preferred embodiment, the electron transfer is detected fluorometrically. Numerous transition metal complexes, including those of ruthenium, have distinct fluorescence properties. Therefore, the change in redox state of the electron donors and electron acceptors attached to the nucleic acid can be monitored very sensitively using fluorescence, for example with Ru(4,7-biphenyl$_2$-phenanthroline)$_3^{2+}$. The production of this compound can be easily measured using standard fluorescence assay techniques. For example, laser induced fluorescence can be recorded in a standard single cell fluorimeter, a flow through "on-line" fluorimeter (such as those attached to a chromatography system) or a multi-sample "plate-reader" similar to those marketed for 96-well immuno assays.

Alternatively, fluorescence can be measured using fiber optic sensors with nucleic acid probes in solution or attached to the fiber optic. Fluorescence is monitored using a photomultiplier tube or other light detection instrument attached to the fiber optic. The advantage of this system is the extremely small volumes of sample that can be assayed.

In addition, scanning fluorescence detectors such as the FluorImager sold by Molecular Dynamics are ideally suited to monitoring the fluorescence of modified nucleic acid molecules arrayed on solid surfaces. The advantage of this system is the large number of electron transfer probes that can be scanned at once using chips covered with thousands of distinct nucleic acid probes.

Many transition metal complexes display fluorescence with large Stokes shifts. Suitable examples include bis- and trisphenanthroline complexes and bis- and trisbipyridyl complexes of transition metals such as ruthenium (see Juris, A., Balzani, V., et. al. Coord. Chem. Rev., V. 84, p. 85-277, 1988). Preferred examples display efficient fluorescence (reasonably high quantum yields) as well as low reorganization energies. These include Ru(4,7-biphenyl$_2$-phenanthroline)$_3^{2+}$, Ru(4,4'-diphenyl-2,2'-bipyridine)$_3^{2+}$ and platinum complexes (see Cummings et al., J. Am. Chem. Soc. 118:1949-1960 (1996), incorporated by reference).

Alternatively, a *reduction* in fluorescence associated with hybridization can be measured using these systems. An electron transfer "donor" molecule that fluoresces readily when on single stranded nucleic acid (with an "acceptor" on th other end) will undergo a reduction in fluor scent intensity when

In a preferred embodiment, alternative electron detection modes are utilized. For example, potentiometric (or voltammetric) measurements involve non-faradaic (no net current flow) processes and are utilized traditionally in pH and other ion detectors. Similar sensors are used to monitor electron transfer through nucleic acid. In addition, other properties of insulators (such as resistance) and of conductors (such as conductivity, impedance and capicitance) could be used to monitor electron transfer through nucleic acid. Finally, any system that generates a current (such as electron transfer) also generates a small magnetic field, which may be monitored in some embodiments.

It should be understood that one benefit of the fast rates of electron transfer observed in the compositions of the invention is that time resolution can greatly ennance the signal-to-noise results of monitors based on absorbance, fluorescence and electronic current. The fast rates of electron transfer of the present invention result both in high signals and stereotyped delays between electron transfer initiation and completion. By amplifying signals of particular delays, such as through the use of pulsed initiation of electron transfer and "lock-in" amplifiers of detection, between two and four orders of magnitude improvements in signal-to-noise may be achieved.

In a preferred embodiment, electron transfer is initiated using alternating current (AC) methods. Without being bound by theory, it appears that nucleic acids, bound to an electrode, generally respond similarly to an AC voltage across a resistor and capacitor in series. Basically, any methods which enable the determination of the nature of these complexes, which act as a resistor and capacitor, can be used as the basis of detection. Surprisingly, traditional electrochemical theory, such as exemplified in Laviron et al., J. Electroanal. Chem. 97:135 (1979) and Laviron et al., J. Electroanal. Chem. 105:35 (1979), both of which are incorporated by reference, do not accurately model the systems described herein, except for very small E_{AC} (less than 10 mV) and relatively large numbers of molecules. That is, the AC current (I) is not accurately described by Laviron's equation. This may be due in part to the fact that this theory assumes an unlimited source and sink of electrons, which is not true in the present systems.

Accordingly, alternate equations were developed, using the Nernst equation and first principles to develop a model which more closely simulates the results. This was derived as follows. The Nernst equation, Equation 1 below, describes the ratio of oxidized (O) to reduced (R) molecules (number of molecules = n) at any given voltage and temperature, since not every molecule gets oxidized at the same oxidation potential.

Equation 8

$$[R] = \frac{1}{1 + \exp^{38.9\,(E - E_0)}} \qquad (5)$$

Taking into consideration the generation of an AC faradaic current, the ratio of [O]/[R] at any given potential must be evaluated. At a particular E_{DC} with an applied E_{AC}, as is generally described herein, at the apex of the E_{AC} more molecules will be in the oxidized state, since the voltage on the surface is now (E_{DC} + E_{AC}); at the bottom, more will be reduced since the voltage is lower. Therefore, the AC current at a given E_{DC} will be dictated by both the AC and DC voltages, as well as the shape of the Nernstian curve. Specifically, if the number of oxidized molecules at the bottom of the AC cycle is subtracted from the amount at the top of the AC cycle, the total change in a given AC cycle is obtained, as is generally described by Equation 9. Dividing by 2 then gives the AC amplitude.

Equation 9

$$i_{AC} = \frac{(\text{electrons at } [E_{DC} + E_{AC}]) - (\text{electrons at } [E_{DC} - E_{AC}])}{2}$$

Equation 10 thus describes the AC current which should result:

Equation 10

$$i_{AC} = C_0\, F\omega\, \tfrac{1}{2}\, ([O]_{E_{DC} + E_{AC}} - [O]_{E_{DC} - E_{AC}}) \qquad (6)$$

As depicted in Equation 11, the total AC current will be the number of redox molecules C), times faraday's constant (F), times the AC frequency (ω), times 0.5 (to take into account the AC amplitude), times the ratios derived above in Equation 7. The AC voltage is approximated by the average, $E_{AC}2/\pi$.

Equation 11

$$i_C = \frac{C_0\, F\omega}{2} \left(\frac{\exp^{38.9\,[E_{DC} + \frac{2E_{AC}}{\pi} - E_0]}}{1 + \exp^{38.9\,[E_{DC} + \frac{2E_{AC}}{\pi} - E_0]}} \right) - \frac{\exp^{38.9\,[E_{DC} - \frac{2E_{AC}}{\pi} - E_0]}}{1 + \exp^{38.9\,[E_{DC} - \frac{2E_{AC}}{\pi} - E}} \qquad (7)$$

Using Equation 11, simulations were generated using increasing overpotential (AC voltage). Figure 22A shows one of these simulations, whil Figure 22B depicts a simulation based on traditional theory.

configurations in the system, including a second electron transfer moiety attached to the target nucleic acid, a second probe nucleic acid containing a second electron transfer moiety, intervening nucleic acids, etc.

In a preferred embodiment, the single stranded nucleic acid is covalently attached to the electrode via a spacer. By "spacer" herein is meant a moiety which holds the nucleic acid off the surface of the electrode. In a preferred embodiment, the spacer is a conductive oligomer as outlined herein, although suitable spacer moieties include passivation agents and insulators as outlined above. The spacer moieties may be substantially non-conductive, although preferably (but not required) is that the rate of electron transfer through the spacer is faster than the rate through single stranded nucleic acid, although substantially conductive spacers are generally preferred. In general, the length of the spacer is as outlined for conductive polymers and passivation agents. Similarly, spacer moieties are attached as is outlined above for conductive oligomers, passivation agents and insulators, for example using the same "A" linker defined herein.

The target sequence is added to the composition under conditions whereby the target sequence, if present, will bind to the probe single stranded nucleic acid to form a hybridization complex, as outlined above.

A first input electrical signal is then applied to the system, preferably via at least the sample electrode (containing the complexes of the invention) and the counter electrode, to initiate electron transfer between the electrode and the second electron transfer moiety. Three electrode systems may also be used, with the voltage applied to the reference and working electrodes. The first input signal comprises at least an AC component. The AC component may be of variable amplitude and frequency. Generally, for use in the present methods, the AC amplitude ranges from about 1 mV to about 1.1 V, with from about 10 mV to about 800 mV being preferred, and from about 10 mV to about 500 mV being especially preferred. The AC frequency ranges from about 0.01 Hz to about 10 MHz, with from about 1 Hz to about 1 MHz being preferred, and from about 1 Hz to about 100 kHz being especially preferred

Surprisingly, the use of combinations of AC and DC signals allows the differentiation between single-stranded nucleic acid and double stranded nucleic acid, as is outlined herein. In addition, signals comprised of AC and DC components also allow surprising sensitivity and signal maximization.

In a pr ferred embodiment, the first input signal comprises a DC component and an AC component. That is, a DC offset voltage between th sample and count r electrodes is swept through th

quantify the actual number of molecules that are on the surface of the electrode. The sample can then be added, an output signal determined, and the ratio of bound/unbound molecules determined. This is a significant advantage over prior methods.

In a preferred embodiment, measurements of the system are taken at at least two separate amplitudes or overpotentials, with measurements at a plurality of amplitudes being preferred. As noted above, changes in response as a result of changes in amplitude may form the basis of identification, calibration and quantification of the system. In addition, one or more AC frequencies can be used as well.

In a preferred embodiment, the AC frequency is varied. At different frequencies, different molecules respond in different ways. As will be appreciated by those in the art, increasing the frequency generally increases the output current. However, when the frequency is greater than the rate at which electrons may travel between the electrode and the second electron transfer moiety, higher frequencies result in a loss or decrease of output signal. For example, as depicted in Figure 11, a response may be detected at 1 Hz for both single stranded nucleic acid and double stranded nucleic acid. However, at the higher frequencies, such as 200 Hz and above, the response of the single stranded nucleic acid is absent, while the response of the double stranded nucleic acid continues to increase. At some point, the frequency will be greater than the rate of electron transfer through even double-stranded nucleic acid, and then the output signal will also drop. Thus, the different frequency responses of single stranded and double stranded nucleic acids, based on the rate at which electrons may travel through the nucleic acid (i.e. the impedance of the nucleic acid), forms the basis of selective detection of double stranded nucleic acids versus single stranded nucleic acids.

In one embodiment, detection utilizes a single measurement of output signal at a single frequency. That is, the frequency response of a single stranded nucleic acid can be previously determined to be very low at a particular high frequency. Using this information, any response at a high frequency, for example such as 10 to 100 kHz, where the frequency response of the single stranded nucleic acid is very low or absent, will show the presence of the double stranded hybridization complex. That is, any response at a high frequency is characteristic of the hybridization complex. Thus, it may only be necessary to use a single input high frequency, and any frequency response is an indication that the hybridization complex is present, and thus that the target sequence is present.

In addition, th us of AC techniques allows the significant reduction of background signals at any single frequency due to entities other than the covalently attached nucleic acids, i.e. "locking out" or "filtering" unwanted signals. That is, the frequency response of a charge carrier or redox active

In a preferred embodiment, the output signal comprises an AC current. As outlined above, the magnitude of the output current will depend on a number of parameters. By varying these parameters, the system may be optimized in a number of ways.

In general, AC currents generated in the present invention range from about 1 femptoamp to about 1 milliamp, with currents from about 50 femptoamps to about 100 microamps being preferred, and from about 1 picoamp to about 1 microamp being especially preferred.

In a preferred embodiment, the output signal is phase shifted in the AC component relative to the input signal. Without being bound by theory, it appears that surprisingly, the systems of the present invention are sufficiently uniform to allow phase-shifting based detection. That is, the complex biomolecules of the invention through which electron transfer occurs react to the AC input in a homogeneous manner, similar to standard electronic components, such that a phase shift can be determined. This may serve as the basis of detection between single-stranded and double stranded nucleic acids, but more importantly, may allow the detection of mismatches, since small changes in impedance, such as would be assumed from a mismatch present in the hybridization complex, may effect the output AC phase in a greater manner than the frequency response.

The output signal is characteristic of electron transfer through the hybridization complex; that is, the output signal is characteristic of the presence of double stranded nucleic acid. In a preferred embodiment, the basis of the detection is a difference in the faradaic impedance of the system as a result of the formation of the hybridization complex. Faradaic impedance is the impedance of the system between the two electron transfer moieties, i.e. between the electrode and the second electron transfer moiety. Faradaic impedance is quite different from the bulk or dielectric impedance, which is the impedance of the bulk solution between the electrodes. Many factors may change the faradaic impedance which may not effect the bulk impedance, and vice versa. Thus, nucleic acids in this system have a certain faradaic impedance, that will depend on the distance between the electron transfer moieties, their electronic properties, and the composition of the intervening medium, among other things. Of importance in the methods of the invention is that the faradaic impedance between the electron transfer moieties is signficantly different depending on whether the intervening nucleic acid is single stranded or double stranded. Thus, the faradaic impedance of the system changes upon the formation of a hybridization complex, and it is this change which is characteristic of the hybridization complex.

Accordingly, th present invention further provides apparatus for the detection of nucleic acids using AC detection methods. The apparatus includes a test chamber which has at least a first measuring or

detected in this way. Bacterial infections such as tuberculosis, clymidia and other sexually transmitted diseases, may also be detected.

In a preferred embodiment, the nucleic acids of the invention find use as probes for toxic bacteria in the screening of water and food samples. For example, samples may be treated to lyse the bacteria to release its nucleic acid, and then probes designed to recognize bacterial strains, including, but not limited to, such pathogenic strains as, *Salmonella*, *Campylobacter*, *Vibrio cholerae*, *Leishmania*, enterotoxic strains of *E. coli*, and Legionnaire's disease bacteria. Similarly, bioremediation strategies may be evaluated using the compositions of the invention.

In a further embodiment, the probes are used for forensic "DNA fingerprinting" to match crime-scene DNA against samples taken from victims and suspects.

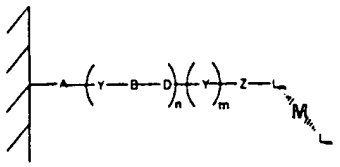
In an additional embodiment, the probes in an array are used for sequencing by hybridization.

The present invention also finds use as a unique methodology for the detection of mutations or mismatches in target nucleic acid sequences. As a result, if a single stranded nucleic acid containing electron transfer moieties is hybridized to a target sequence with a mutation, the resulting perturbation of the base pairing of the nucleosides will measurably affect the electron transfer rate. This is the case if the mutation is a substitution, insertion or deletion. Alternatively, two single stranded nucleic acids each with a covalently attached electron transfer species that hybridize adjacently to a target sequence may be used. Accordingly, the present invention provides for the detection of mutations in target sequences.

Thus, the present invention provides for extremely specific and sensitive probes, which may, in some embodiments, detect target sequences without removal of unhybridized probe. This will be useful in the generation of automated gene probe assays.

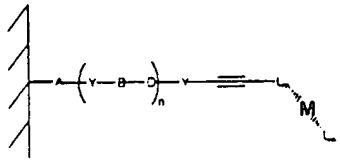
In an alternate embodiment the electron transfer moieties are on separate strands. In this embodiment, one single stranded nucleic acid has an electrode covalently attached via a conductive oligomer. The putative target sequences are labelled with a second electron transfer moiety as is generally described herein, i.e. by incorporating an electron transfer moiety to individual nucleosides of a PCR reaction pool. Upon hybridization of the two single-stranded nucleic acids, electron transfer is detected.

Structure 35

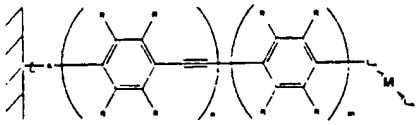


Structure 35 utilizes a Structure 4 conductive oligomer, although as will be appreciated by those in the art, other conductive oligomers such as Structures 2, 3, 9 or 10 types may be used. Preferred embodiments of Structure 35 are depicted below.

Structure 36

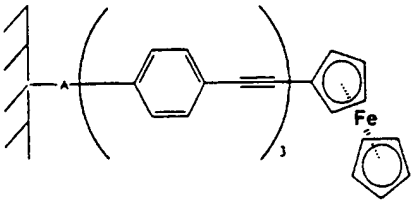


Structure 37



Preferred R groups of Structure 37 are hydrogen.

Structure 38



These compositions are synthesized as follows. The conductive oligomer linked to the metallocene is made as described herein; see also, Hsung et al., Organometallics 14:4808-4815 (1995); and Bumm et al., Science 271:1705 (1996), both of which are expressly incorporated herein by reference. The conductive oligomer is then attached to the electrode using the novel ethylpyridine protecting group, as outlined herein.

Compound # 2: To a solution of 3.4 gm (9.97 mmol) of **Compound # 1** in 70 mL of diethylamine was added 200 mg of bis(triphenylphosphine)palladium (II) chloride, 100 mg of cuprous iodide and 1.9 mL of trimethylsilylacetylene under Argon. The reaction mixture was stirred for 2 h. After removing the diethylamine, the residue was dissolved in dichloromethane for column chromatography. Silica gel (120 gm) was packed with a cosolvent of 50 % ethyl acetate / 50 % hexane. The crude sample solution was loaded and the column was eluted with the same cosolvent. After removing the solvents, the liquid title compound (2.6 gm, 83.7 %) was obtained.

Compound # 3: To a solution of 2.6 gm of **Compound # 2** in 150 mL of dichloromethane colled in an ice-water bath was added 9.0 mL of 1 N tetrabutylammonium fluoride THF solution. The reaction mixture was stirred for 1 h. and washed once with water and dried over anhydrous Na_2SO_4. After removing the solvent, the residue was used for column separation. Silica gel (50 gm) was packed with a coslovent of 50 % ethyl acetate / 50 % hexane. The crude product solution was loaded and the column was eluted with the same solvents. The removal of the solvents gave the solid title compound (1.87 gm, 94.1 %).

Compound # 4: To a glass bottle were added 1.80 gm (7.52 mmol) of **Compound # 3**, 160 mg of bis(triphenylphosphine)palladium (II) chloride, 80 mg of cuprous iodide and 2.70 gm (9.0 mmol) of 1-trimethylsilyl-2-(4-iodophenyl)acetylene. The bottle was sealed and bubbled with Argon. Diethylamine was introduced by a syringe. The reaction mixture was heated at 50 °C under Argon for 1h. The amine was removed and the residue was dissolved in dichloromethane for the separation. Silica gel (100 gm) was packed with 60 % ethyl acetate / hexane. The crude mixture was loaded and the column was eluted with the same solvents. The fractions were identified by TLC (EtOAc : Hexane = 50 : 50, the product emitted blue light) and pooled. The removal of the solvents gave the solid title product (2.47 gm, 79.8 %).

Compound # 5: To a solution of 2.47 gm of **Compound # 4** in 130 mL of dichloromethane cooled in an ice-water bath was added 8.0 mL of 1 N tetrabutylammonium fluoride THF solution. The reaction mixture was stirred for 1 h. and washed once with water and dried over anhydrous Na_2SO_4. After removing the solvent, the residue was used for column separation. Silica gel (60 gm) was packed with a coslovent of 50 % ethyl acetate / 50 % CH_2Cl_2. The crude solution was loaded and the column was eluted with the same solvents. The removal of solvents gave the solid title product (1.95 gm, 95.7 %).

Comp und # 6: To a glass bottle were added 0.23 gm (0.68 mmol) of **Compound # 5**, 0.5 gm (0.64 mmol) of 2'-deoxy-2'-(4-iodophenylcarbonyl) amino-5'-O-DMT uridine, 60 mg of bis(triphenylphosphine)palladium (II) chloride, 30 mg of cuprous iodid . The bottle was sealed and

1 h. To the prepared solution were added 0.1 gm of DMAP and 3.0 gm (10.9 mmol) 4-iodobezoyl chloride and the reaction mixture was stirred overnight. To this solution was added 30 mL of concentrated ammonium hydroxide solution and the mixure was stirred for exact 15 min. The solvents were removed in vacuo. The residue was dissolved in 15 mL of dichloromethane for column separation. Silica gel (125 gm) was packed with 1 % TEA/2 % CH_3OH/CH_2Cl_2. After loading the sample, the column was eluted with 300 mL of 1 % TEA/2 % CH_3OH/CH_2Cl_2, and 500 mL of 1 % TEA/4 % CH_3OH/CH_2Cl_2. The fractions were identified by TLC (CH_3OH : CH_2Cl_2 = 10 : 90) and pooled and concentrated to dryness to give 6.2 gm (85.5 %) of the pure title compound.

Synthesis of the Phosphormidite (Compound # 8).

To a solution of 0.2 gm of Compound # 6 and 30 mg of diisopropylammonium tetrazolide in 10 mL of dry dichloromethane is added 0.12 gm of 2-cyanoethyl N, N, N', N'-tetraisopropylphosphane under Argon. The solution was stirred for 5 h and diluted by adding 60 mL of dichloromethane. The solution was washed twice with 2.5 % w/v sodium bicarbonate solution, once with the brine and dried over sodium sulfate. After removing the solvent, residue was dissolved in 5 mL of dichloromethane, followed by adding slowly 100 mL of hexane. The suspension was stored at - 20 °C for 1 h. The supernatant was decanted and the residue was dried over a high vacuum overnight to afford 0.19 gm (79.0 %) of the title product, which will be used for DNA synthesis.

In addition, this procedure was done to make a four unit wire.

Example 2

Synthesis of conductive oligomers linked to the ribose of a nucleoside via an amine linkage

Example 2A:

Synthesis of 2'-(4-iodophenyl)amino-2'-deoxy-5'-O-DMT-uridine (Product 4): This synthesis is depicted in Figure 2, and reference is made to the labelling of the products on the figure. To a solution of 5.0 gm of 5'-O-DMT-uridine (**Product 1**) and 2.7 gm of dimethylaminopyridine in 200 mL of acetonitrile was added 3.3 gm of p-iodophenyl isocyalide dichloride portion by portion under Argon. The reaction mixture was stirred overnight. The mixture was diluted by adding 550 mL of dichloromethane and washed twice with 5 % sodium bicarbonate aqueous solution and once with the brine solution, and then dried over sodium sulfate. The removal of the solvent in vacuo gave the crude **Product 2**. Without further purification, Product 2 was dissolved in 50 mL of dry DMF and the solution was heated at 150 °C foe 2 h. After distillation of DMF, the residue was dissolved in 300 mL of dichloromethane, washed once with 5 % sodium bicarbonate solution, once with the brine solution

and the organic layer was separated and washed once with the 500 mL of the brine solution and dried over sodium sulfate. The dichloromethane was removed by a rotavapor and the dixoxane was removed by a high vacuum. The residue was dissolved in 20 mL of dichloromethane for the separation. Silica gel (80 gm) was packed with 1 % TEA / 25 % EtOAc / CH_2Cl_2 and the sample solution was loaded. The column was eluted with 1 % TEA / 25-50 % EtOAc / CH_2Cl_2. The right fractions were combined and concentrated to give 4.1 gm (65.7 %) of the final product **C4**.

Example 3

Synthesis of a conductive oligomer with an R group attached to the Y aromatic group

This synthesis is depicted in Figure 6.

Synthesis of 2-Acetyl-5-iodotoluene (P 1). To a suspension of 20 gm of aluminum trichloride in 500 mL of dichloromethane was added 10.2 mL of acetyl chloride under Argon. After the reaction mixture was stirred for 15 min, 3-iodotoluene (20 gm) was added through a syringe. The mixture was stirred overnight under Argon and poured into 500 gm of ice-water. Organic layer was separated and washed once with the saturated ammonium chloride solution, and washed once with 10 % sodium thiosulfate solution and dried over sodium sulfate. After removing the solvent, the residue was dissolved in hexane for the column purification. Silica gel (260 gm) was packed with hexane, after loading the sample solution, the column was eluted with 750 mL of hexane, 750 mL of 1 % v/v ether / hexane, 750 mL of 2 % v/v ether / hexane and 1500 mL of 3 % v/v ether / hexane. The fractions containing the right isomer were identified by GC-MS and ^1H NMR and pooled and concentrated to dryness to afford 12.2 gm (51.2 %) of the title product (**P 1**).

Iodo-3-methyl-4-(ehynyl trimethylsilyl) benzene (P2). Under inert atmosphere 500 ml bound bottom flask was charged with 25 ml of dry THF, cooled to -78°C and 14 ml of 2.0 M LDA solution (heptane/ethylbenzene/ THF solution) was added by syringe. To this solution 6.34 gr (24.38 mmole) of iodo-3-methyl-4-acetyl benzene in 25 ml of THF was added dropwise and the reaction mixture was stirred for 1 hr at -78°C, then 4.0 ml (19.42 mmole) of diethylchlorophosphate were added by syringe. After 15 min cooling bath was removed and the reaction mixture was allowed to heat up to RT and stirred for 3 hrs. The resulted mixture was cooled again to -78°C and 29 ml of 2.0 M LDA solution were added dropwise. At the end of the addition the reaction mixture was allowed to warm up to RT and stirred for additional 3 hrs. After that period of time it was cooled again to -20°C, 9.0 ml (70.91 mmole) of trimethylsilyl chloride were injected and the stirring was continued for 2 hrs at RT. The reaction mixture was poured into 200 ml of ice/sodium bicarbonate saturated aqueous solution and 300 ml of ether were added to extract organic compounds. The aqueous phas was separated and

Other conductive oligomers with R groups are depicted in Figure 17, which were made using the techniques outlined herein.

Example 4

Synthesis of a nucleoside with a metallocene second electron transfer moiety attached via a ribose

Synthesis of **5'-O-DMT-2'-deoxy-2'-(ferrocenecarbonyl)amino Uridine (UAF):** To a solution of 2.5 gm(10.9 mmol) of ferrocene monocarboxylic acid in 350 mL of dichloromethane were added 2.25 gm (10.9 mmol) of DCC and 1.27 gm (10.9 mmol) of N-hydroxysuccinimide. The reaction mixture was stirred for 3 h and the precipitate was formed. The precipitate was filtered off and washed once with dichloromethane. The combined filtrate was added into 4.5 gm (8.25 mmol) of 2'-deoxy-2'-amino-5'-O-DMT uridine, followed by adding 2 mL of triethylamine. The reaction mixture was stirred at room temperature for 8 days. After removing the solvent, the residue was dissolved in dichloromethane for separation. Silica gel (120 gm) was packed with 1 % TEA / 2 % CH_3OH / CH_2Cl_2. After loading the sample solution, the column was eluted with 2-7 % CH_3OH/1 % TEA / CH_2Cl_2. The fraction was identified by TLC(CH_3OH : CH_2Cl_2 = 1 : 9) and pooled and concentrated to dryness to afford 1.3 gm(22.0 %) of the title compound.

Synthesis of **UAF Phosphoramidite**:

Preparation of **Diisopropylaminochloro(β-cyano)ethoxyphosphine**: To a solution of 0.54 mL(4.0 mmol) of dichloro(β-cyano)ethoxyphosphine in 40 mL of dichloromethane cooled in an ice-water bath was added 10 mL of diisopropylethylamine, followed by adding 0.64 mL (4.0 mmol) of diisopropylamine under Argon. The reaction mixture was warmed up to room temperature and stirred for 2 h. After adding 0.1 gm of DMAP into the solution, the reaction mixture is ready for the next step reaction.

Preparation of UAF phosphoramidite: To a solution of 1.30 gm (1.72 mmol) of 5'-O-DMT-5-ferrocenylacetylenyl-2'-deoxy uridine in 40 mL of dichloromethane cooled in an ice-water bath was added 10 mL of diisopropylethylamine. The prepared phosphine solution was transferred into the nucleoside solution through a syringe. The reaction mixture was warmed up to room temperature and stirred overnight. The solution was diluted by adding 100 mL of dichloromethane and washed once with 200 mL od 5 % aqueous $NaHCO_3$ solution, and once with the brine (200 mL) and dried over Na_2SO_4 and concentrated to dryness. Silica gel(47 gm) was packed with 2 % TEA/1 % CH_3OH/CH_2Cl_2. The residue was dissolved in 10 mL of dichloromethane and loaded. The column

Preparation of **UBF phosphoramidite:** To a solution of 3.42 gm (4.63 mmol) of 5'-O-DMT-5-ferrocenylacetylenyl-2'-deoxy uridine in 40 mL of dichloromethane cooled in an ice-water bath was added 10 mL of diisopropylethylamine. The prepared phosphine solution was transferred into the nucleoside solution through a syringe. The reaction mixture was warmed up to room temperature and stirred overnight. The solution was diluted by adding 150 mL of dichloromethane and washed once with 200 mL of 5 % aqueous $NaHCO_3$ solution, and once with the brine (200 mL) and dried over Na_2SO_4 and concentrated to dryness. Silica gel(92 gm) was packed with 2 % TEA/1 % CH_3OH/CH_2Cl_2. The residue was dissolved in 10 mL of dichloromethane and loaded. The column was eluted with 500 mL of 1 % TEA/2 % CH_3OH/CH_2Cl_2. The fractions were pooled and concentrated to give 3.0 gm (69.0 %) of the title compound.

Nucleotides containing conductive oligomers and second electron transfer moieties were incorporated into nucleic acids using standard nucleic acid synthesis techniques; see "Oligonucleotides and Analogs, A Practical Approach", Ed. By F. Eckstein, Oxford University Press, 1991, hereby incorporated by reference.

Example 6

Synthesis of an electrode containing nucleic acids containing conductive oligomers with a monolayer of $(CH_2)_{16}$

Using the above techniques, and standard nucleic acid synthesis, the uridine with the phenyl-acetylene conductive polymer of Example 1 was incorporated at the 3' position to form the following nucleic acid: ACCATGGACTCAGCU-conductive polymer of Example 1 (hereinafter "wire-1").

HS-(CH2)16-OH (herein "insulator-2") was made as follows.

16-Bromohexadecanoic acid. 16-Bromohexadecanoic acid was prepared by refluxing for 48 hrs 5.0 gr (18.35 mmole) of 16-hydroxyhexadecanoic acid in 24 ml of 1:1 v/v mixture of HBr (48% aqueous solution) and glacial acetic acid. Upon cooling, crude product was solidified inside the reaction vessel. It was filtered out and washed with 3x100 ml of cold water. Material was purified by recrystalization from n-hexane, filtered out and dried on high vacuum. 6.1 gr (99% yield) of the desired product were obtained.

16-M rcapt hexad canoic acid. Under inert atmospher 2.0 gr of sodium metal suspension (40% in mineral oil) were slowly added to 100 ml of dry methanol at 0°C. At the end of the addition reaction mixture was stirred for 10 min at RT and 1.75 ml (21.58 mmole) of thioacetic acid were added. After

with ethanol and dried. 20-30 microliters of wire-1 solution (1 micromolar in 1XSSC buffer at pH 7.5) was applied to the electrode in a round droplet. The electrode was incubated at room temperature for 4 hours in a moist chamber to minimize evaporation. The wire-1 solution was then removed from the electrode and the electrode was immersed in 1XSSC buffer followed by 4 rinses with 1XSSC. The electrode was then stored at room temperature for up to 2 days in 1XSSC.

Alternatively, and preferably, either a "two-step" or "three-step" process is used. The "two-step" procedure is as follows. The wire-1 compound, in water at ~ 5-10 micromolar concentration, was exposed to a clean gold surface and incubated for ~ 24 hrs. It was rinsed well with water and then ethanol. The gold was then exposed to a solution of ~ 100 micromolar insulator thiol in ethanol for ~ 12 hrs, and rinsed well. Hybridization was done with complement for over 3 hrs. Generally, the hybridization solution was warmed to 50°C, then cooled in order to enhance hybridization.

The "three-step" procedure uses the same concentrations and solvents as above. The clean gold electrode was incubated in insulator solution for ~ 1 hr and rinsed. This procedure presumably results in an incomplete monolayer, which has areas of unreacted gold. The slide was then incubated with wire-1 solution for over 24 hrs (generally, the longer the better). This wire-1 still had the ethyl-pyridine protecting group on it. The wire-1 solution was 5% NH4OH, 15% ethanol in water. This removed the protecting group from the wire and allowed it to bind to the gold (an in situ deprotection). The slide was then incubated in insulator again for ~ 12 hrs, and hybridized as above.

In general, a variety of solvent can be used including water, ethanol, acetonitrile, buffer, mixtures etc. Also, the input of energy such as heat or sonication appears to speed up all of the deposition processes, although it may not be necessary. Also, it seems that longer incubation periods for both steps, for example as long as a week, the better the results.

Hybridization efficiency was determined using ^{32}P complementary and noncomplementary 15 mers corresponding to the wire-1 sequence. The electrodes were incubated with 50 microliters of each of the labelled non-complementary (herein "A5") or complementary (herein "S5") target sequences applied over the entire electrode in 1XSSC as depicted in Table 1. The electrodes were then incubated for 1-2 hours at room temperature in a moist chamber, and rinsed as described above. The amount of radiolabelled DNA was measured for each electrode in a scintillation counter, and the electrodes were dried and exposed to X-ray film for 4 hours.

Compound #12. To 200 mg (0.32 mmole) of suspension of MG#1 in 200 ml of acetone (sonication was applied in order to get better results) 3 ml of MeI were added and the reaction mixture was stirred for 20 hrs at RT. After that time volume of the resulted solution was reduced by rotovap evaporation to 50 ml and then 400 ml of n-hexane were added. Formed precipitate was filtered out, washed with 3x200 ml of n-hexane and dried on high vacuum. Quantitative yield of the desired compound was obtained.

Compound #13. To 100 mg (0.13 mmole) of suspension of MG#2 in 200 ml of acetone (sonication was applied in order to get better results) 10 ml of triethyl amine were added and the reaction mixture was stirred for 20 hrs at RT. After that time volume of the resulted solution was reduced by rotovap evaporation to 50 ml and then 400 ml of n-hexane were added. Formed precipitate was filtered out, washed with 3x200 ml of n-hexane and dried on high vacuum. The desired compound was extracted from this precipitate with 3x50 ml of THF. Evaporation of the THF fractions gave 35 mg (52%) of the compound #13. This was then added to a gold electrode as known in the art.

HS-(CH2)15NHCO-Fc (herein "insulator-1") was made as described in Ward et al., Anal. Chem. 66:3164-3172 (1994), hereby incorporated by reference (note: the Figure 1 data has been shown to be incorrect, although the synthesis of the molecule is correct).

Monolayers of each were made as follows. Insulator: Gold covered microscope slides were immersed in a mixture of insulator-1 and HS-(CH2)15-OH (insulator-2) in neat ethanol. Insulator-2 molecule is added to the mixture to prevent the local concentration of ferrocene at any position from being too high, resulting in interactions between the ferrocene molecules. The final solution was 0.1 mM insulator-1 and 0.9 mM insulator-2. The mixture was sonicated and heated (60-80°C) for 1-10 hours. The electrodes were rinsed thoroughly with ethanol, water and ethanol. The electrodes were immersed in a 1 mM thiol solution in neat ethanol and let stand at room temperature for 2-60 hours. The electrodes were then rinsed again. This procedure resulted in 1-10% coverage of insulator-1 as compared to calculated values of close packed ferrocene molecules on a surface. More or less coverage could easily be obtained by altering the mixture concentration and/or incubation times.

Wires: The same procedure was followed as above, except that the second step coating required between 10 and 60 hours, with approximately 24 hours being preferable. This resulted in lower coverages, with between 0.1 and 3% occurring.

Cyclic voltametry was run at 3 scan speeds for each compound: 1V/sec, 10 V/ec, and 50 V/s c. Ev n at 1 V/sec, significant splitting occurs with insulator-1, with roughly 50 mV splitting occuring. At higher

similar surface melted at approximately 45°C. Repeating the scan after the heat treatment shows a reduced signal, as in the first scan prior to hybridization.

Example 9

AC detection methods

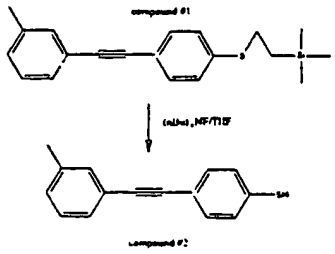
Electrodes containing four different compositions of the invention were made and used in AC detection methods. In general, all the electrodes were made by mixing a ratio of insulator-2 with the sample as is generally outlined above.

Sample 1, labeled herein as "Fc-alkane", contained a mixed monolayer of insulator-2 and insulator-1. Sample 2, labeled herein as "Fc-amido-alkane", contained a mixed monolayer of insulator-2 and a derivative of insulator-1 which has an amido attachment of the ferrocene to the alkane. Sample 3, labeled herein as "Fc-wire", contained a mixed monolayer of insulator-2 and wire-2. Sample 4 was the same as Sample 3, with the exception that a new in situ deprotection step was used, described below. Sample 5, labeled herein as "ssDNA" (AGCTGAGTCCA(UBF)GGU-conductive oligomer), contained a mixed monolayer of insulator-2 and wire-3. Sample 6, labeled herein as "dsDNA", contained a mixed monolayer of insulator-2 and wire-3, wherein the complement of wire-3 was hybridized to form a double stranded wire-3. Sample 7 was a solution of ferrocene in solution. As is shown herein, the rat of electron transfer, from fast to slower, is as follows: Sample 3 > Sample 6 > Sample 1 > Sample 2 > Sample 5. Generally, Sample 1 models ssDNA, and Sample 3 models dsDNA.

The experiments were run as follows. A DC offset voltage between the working (sample) electrode and the reference electrode was swept through the electrochemical potential of the ferrocene, typically from 0 to 500 mV. On top of the DC offset, an AC signal of variable amplitude and frequency was applied. The AC current at the excitation frequency was plotted versus the DC offset.

Figure 8 depicts an experiment with Sample 1, at 200 mV AC amplitude and frequencies of 1, 5 and 100 Hz. Sample 1 responds at all three frequencies, and higher currents result from higher frequencies, which is simply a result of more electrons per second being donated by the ferrocene at higher frequencies. The faster the rate, the higher the frequency response, and the better the detection limit. Figure 9 shows overlaid AC voltammograms of an electrode coated with Sample 3. Four excitation frequencies were applied: 10 Hz, 100 Hz, 1 kHz, 10 kHz, all at 25 mV overpotential. Figure 10, shows the frequency response of samples 1, 2 and 3 by measuring the peak currents vs. frequency. Sample 3 response to increasing frequencies through 10 kHz (the detector system limit), while Sample 1 lose its responses at between 20 and 200 Hz. Thus, to discriminate between Sample

The use of an alternate protecting group for protection of the sulfur atom prior to attachment to the gold surface was explored.



To 0.5 gm of molecular sieve (3 Å) was added 3 ml of dry THF and 2.5 ml of 1.0 tetrabutylammonium fluoride. After stirring for 20 minutes, 100 mg of compound #1 was added under Argon. The reaction mixture was stirred for 1 hour and poured into 100 ml of 5% citric acid solution and the aqueous solution was shaken well and extracted twice with either (2 X 100 ml). The combined ether solution was dried over Na_2SO_4 and concentrated. The residue was purified by column chromatography using 10% CH_2Cl_2/Hexane as eluent. The purified product was analyzed by ^{1}HNMR which should 50% of compound #2 and 50% of the corresponding disulfide.

The use of this protecting group in synthesizing base-attached conductive oligomers is depicted in Figures 20 and 21.

Example 12

Preparation of Peptide Nucleic Acids with Electron Transfer Moieties

The synthesis of a peptide nucleic acid monomeric subunit with a conductive oligomer covalently attached to the α-carbon is depicted in Figure 31.

4-Iodophenylalanine: Into a solution of 40.15 gm (0.243 mol) of phenylalanine in a mixture of 220 mL of acetic acid and 29 mL of concentrated H_2SO_4 was added 24.65 gm (0.097 mol) of powered iodine and 10. 18 gm(0.051 mol) of powered $NaIO_3$ while stirring. The reaction mixture was stirred at 70 °C for 21 h, during this time, two portions of 1 gm of NaIO3 were added. The mixture was cooled and the acetic acid was removed by using rotavapor while temperature was maintained at 35°C and the residue oil was diluted by adding 400 mL of water. The aqueous solution was extracted once with 100 mL ether and once with 100 of dichloromethane. After decolorization with 5 gm of Norit, the aqueous solution was neutralized by adding solid NaOH to precipitate the crud product, which, after chilling, was filtered and rinsed with 800 mL of water and 300 mL of ethanol. The wet product was recrystallized from 200 mL of acetic acid to produce 37.5 gm of 4-iodo-L-phenylalanine.

Methyl N-(2-MMT-aminoethyl)-N-(2-Nitrobenzenesulfonyl)-4-Iodophenyl Alaninate: To a solution of 16.5 gm (49.5 mmol) of 2-MMT-amino ethanol, 20.0 gm (40.8 mmol) of methyl N-(2-nitrobenzensulfonyl)-4-iodophenyl alaninate and 13 gm (49.5 mmol) of triphenylphosphine in 250 mL of dry THF cooled in an ice-water bath was added 7.8 mL (49.5 mmol) of diethyl azodicarboxylate under Argon. The solution was warmed up to room temperature and stirred overnight. After removing THF, the residue dissolved in the small amount of the CH_2Cl_2 for column separation. TLC (CH_2Cl_2: Hexane = 9:1) of the sample solution indicated two products, i.e., the early spot is the desired product, the later spot is triphenylphosphine oxide. Silica gel (300 gm) was packed with 1% TEA/hexane. The sample solution was loaded and the column was eluted with 500 mL of 1% TEA/hexane, 100 mL of 1% TEA/25% CH_2Cl_2/hexane and 1000 mL of 1% TEA/50% CH_2Cl_2/hexane. The fractions were identified by TLC (CH_2Cl_2:Hexane = 9:1). The fractions containing the pure early spot were pooled and concentrated to give 17 gm of the title compound. The overlapping fractions were pooled, concentrated and repurified to give another 3.0 gm of the title compound. The total yield is 62.0%.

Methyl N-(2-MMT-aminoethyl)-4-iodophenyl Alaninate: To a suspension of 17.0 gm (21 mmol) of methyl N-(2-MMT-aminoethyl)-N-(2nitrobenzenesulfonyl)-4-iodophenyl alaninate, 11.6 gm (84 mmol) of Potassium Carbonate in 150 mL of DMF was added 2.6 mL (25.8 mmol) of thiophenol under Argon. The reaction mixture was stirred at room temperature for 1.3 h. and diluted by adding 1.2 L of the brine. The aqueous solution was extracted three times by ether (2x 500 mL) and the combined extracts was washed once with the diluted NaOH solution and dried over sodium sulfate. After the removal of the solvent, the residue was used for column separation. Silica Gel (220 gm) was packed with 1% TEA/hexane, upon loading the sample solution, the column was eluted with 500 mL of 1% TEA/hexane, 1000 mL of 1% TEA/25% ether/hexane, and 1000 mL of 1% TEA/50% ether/hexane. The fractions were identified by TLC (Ether:Hexane) and pooled and concentrated to afford 5.6 gm (43.1%) of the title product.

Methyl N-(2-MMT-aminoethyl)-N-[(Thymin-1-yl)acetyl]-4-Iodophenyl Alaninate: To a solution of 3.37 g (5.43 mmol) of methyl N-(2-MMT-aminoethyl)-4-iodophenyl alaninate in DMF (10 mL) was added 3,4-Dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (.884 g, 5.43 mmol) and 4-ethylmorpholine (1.38 mL, 10.86 mmol). A solution of thymine acetic acid (1.00 g, 5.43 mmol) in DMF (10 mL) was then added, followed by N,N'-diisopropylcarbodiimide (1 mL, 6.5 mmol). The reaction mixture was left stirring overnight at room temperature for 20.5 h. The solvent was removed in vacuo. The residue was dissolved in 600 mL of CH_2Cl_2 and the solution was washed with twice with 500 mL of water and once with 500 mL of brine and dried in Na_2SO_4. After the removal of the solvent, the crude residue was dissolved in ~10mL of CH_2Cl_2 for column separation. Silica gel (135 gm) was packed with 1% TEA/CH_2Cl_2, upon loading the sample solution, and the column was eluted with 1% TEA/CH_2Cl_2. The

Example 13

Preparation of Peptide Nucleic Acids with Electron Transfer Moieties

The synthesis of a peptide nucleic acid monomeric subunit with a ferrocene electron transfer moiety covalently attached to the base is depicted in Figure 32.

Synthesis of Y1: 5-Iodo uracil (100.0 gm) was suspended in 250 ml of dry DMF. 1.68 gms of sodium hydride was added in portions. The reaction mixture was then stirred at room temperature for 40 minutes. Then 6.16 ml of t-butyl bromoacetate ws added and the reaction mixture was stirred for an additional two hours at room temperature. The reaciton mixture was quenched with 5 ml of methanol containing CO^2. The solvent was then removed and the residue was dissolved in dichloromethane and washed with water. The precipitate was formed during the wash and then filtered and dried. The reaction yielded 9.33 g of product **Y1**.

Synthesis of Y2: To a solution of 6.33 g of **Y1** in 140 ml of dichloromethane was added 35 ml of triethylamine, 0.55 g of 4-dimethylaminopyridine, and 5.89 g of 2-mesitylenesulfonyl chloride. The reaction mixture was stirred for 40 minutes and then 0.40 g of 1.4-diazobicyclo[2,2,2] octane and 4.34 ml of 2,4-dimethylphenol were added and stirred for 2 hours. The reaction mixture was then diluted by adding 200 ml of dichloromethane and the solution was washed with a 5% sodium bicarbonate solution, dried over sodium sulfate and concentrated. The residue was dissolved in 5 ml of dichloromethane and loaded onto a 200 g silica gel column packed with dichloromethane. The column was eluted with 1-5% methanol/dichloromethane. The fractions containing the diesired product was pooled and concentrated to give 2.5 g of **Y2**.

Synthesis of Y3: A mixture of 2.5 g of **Y2**, 1.38 g of ferrocene acetylene, 200 mg of $Pd(pph_3)Cl_2$ and 208 mg of copper iodide in 100 ml of dimehtylformamide (DMF) and 100 ml of triethylamine was degassed well and stirred at 55°C for 2 hours. Upon removing solvent the residue was dissolved in dichloromethane and the solution was washed with a 5% sodium bicarbonate solution, dried over sodium sulfate and concentrated. The crude residue was dissolved in 5 ml of dichloromethane and loaded onto a 200 g silica gel column packed with dichloromethane. The column was eluted with 2-5% methanol/CH_2Cl_2. The right fractions were pooled and evaporated to yield 2.98 g of **Y3**.

Synthesis of Y4: To a solution of 2.50 g of **Y3** in 40 ml of dichloromethane cooled in an ice bath was added 7.1 ml of trimethylsilane, followed by adding 17.5 ml of trifluoroacetic acid. The resulting reaction mixture was warmed to room temperature after 5 min of stirring at the same temperature The reaction mixture was stirred at room temp for 7.5 hours. The solvent was removed. The residue was

CLAIMS

We claim:

1. A composition comprising:

a) a first electron transfer moiety comprising an electrode;

b) a first single stranded nucleic acid;

c) a second electron transfer moiety covalently attached to said first nucleic acid; and

d) a conductive oligomer covalently attached to both said electrode and said first nucleic acid.

2. A composition comprising:

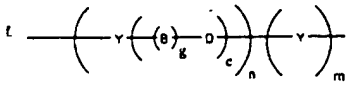
a) a first electron transfer moiety comprising an electrode;

b) a first single stranded nucleic acid;

c) a conductive oligomer covalently attached to both said electrode and said first nucleic acid; and

d) a second electron transfer moiety covalently attached to a second single stranded nucleic acid.

3. A composition according to claim 1 or 2 wherein said conductive oligomer has the formula:



wherein

Y is an aromatic group;

n is an integer from 1 to 50;

g is either 1 or zero;

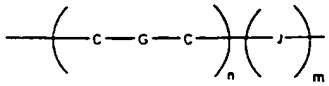
e is an integer from zero to 10;and

m is zero or 1;

wherein when g is 1, B-D is a conjugated bond; and

wherein when g is zero, e is 1 and D is preferably carbonyl, or a heteroatom moiety, wherein the heteroatom is selected from oxygen, sulfur, nitrogen, silicon or phosphorus.

4. A composition according to claim 1 or 2 wherein said conductive oligomer has the formula:



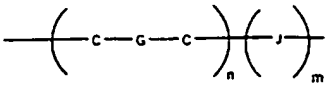
wherein

n is an integer from 1 to 50;

m is 0 or 1;

C is carbon;

11. A method according to claim 9 wherein said conductive oligomer has the formula:



wherein

n is an integer from 1 to 50;

m is 0 or 1;

C is carbon;

J is carbonyl or a heteroatom moeity, wherein the heteroatom is selected from the group consisting of oxygen, nitrogen, silicon, phosphorus, sulfur; and

G is a bond selected from alkane, alkene or acetylene.

12. A method according to claim 9, 10 or 11 wherein said first input signal is selected from the group consisting of applied potential and photoactivation.

13. A method according to claim 9, 10, 11 or 12 wherein said first input signal comprises an AC component and a non-zero DC component.

14. A method according to claim 9, 10, 11, 12 or 13 wherein said first input signal comprises an AC component at a first frequency and a non-zero DC component, and said method further comprises applying a second input signal comprising an AC component at at least a second frequency and a non-zero DC component.

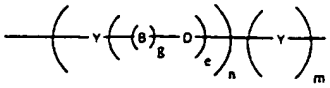
15. A method according to claim 9, 10, 11, 12, 13 or 14 wherein said first input signal comprises an AC component and a first non-zero DC component, and said method further comprises applying a second input signal comprising an AC component and a second non-zero DC component.

16. A method according to claim 9, 10, 11, 12, 13, 14 or 15 wherein said first input signal comprises an AC component at a fist voltage amplitude and said method further comprises applying a second input signal comprising an AC component at a second voltage amplitude.

17. A method according to claim 9, 10, 11, 12, 13, 14, 15 or 16 wherein said input signal includes the use of a co-redoxant.

18. A method according to claim 9, 10, 11, 12, 13, 14, 15, 16 or 17 wherein said input signal includes the use of a hybridization indicator.

b) a nucleic acid comprising at least one nucleoside, wherein said nucleic acid is covalently attached to said solid support with a linker selected from the group selected from:

i) 

wherein

Y is an aromatic group;

n is an integer from 1 to 50;

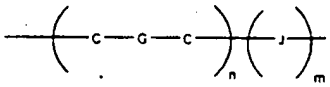
g is either 1 or zero;

e is an integer from zero to 10;and

m is zero or 1;

wherein when g is 1, B-D is a conjugated bond; and

wherein when g is zero, e is 1 and D is preferably carbonyl, or a heteroatom moiety, wherein the heteroatom is selected from oxygen, sulfur, nitrogen, silicon or phosphorus; and

ii) 

wherein

n is an integer from 1 to 50;

m is 0 or 1;

C is carbon;

J is carbonyl or a heteroatom moiety, wherein the heteroatom is selected from the group consisting of oxygen, nitrogen, silicon, phosphorus, sulfur; and

G is a bond selected from alkane, alkene or acetylene, wherein if m = 0, at least one G is not alkane.

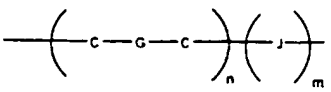
23. A composition according to claim 21 or 22 further comprising a hybridization indicator.

24. A composition comprising:

a) an electrode;

b) at least one metallocene; and

c) a conductive oligomer covalently attached to both said electrode and said metallocene, wherein said conductive oligomer is selected from the group consisting of:

i) 

wherein

n is an integer from 1 to 50;

m is 0 or 1;

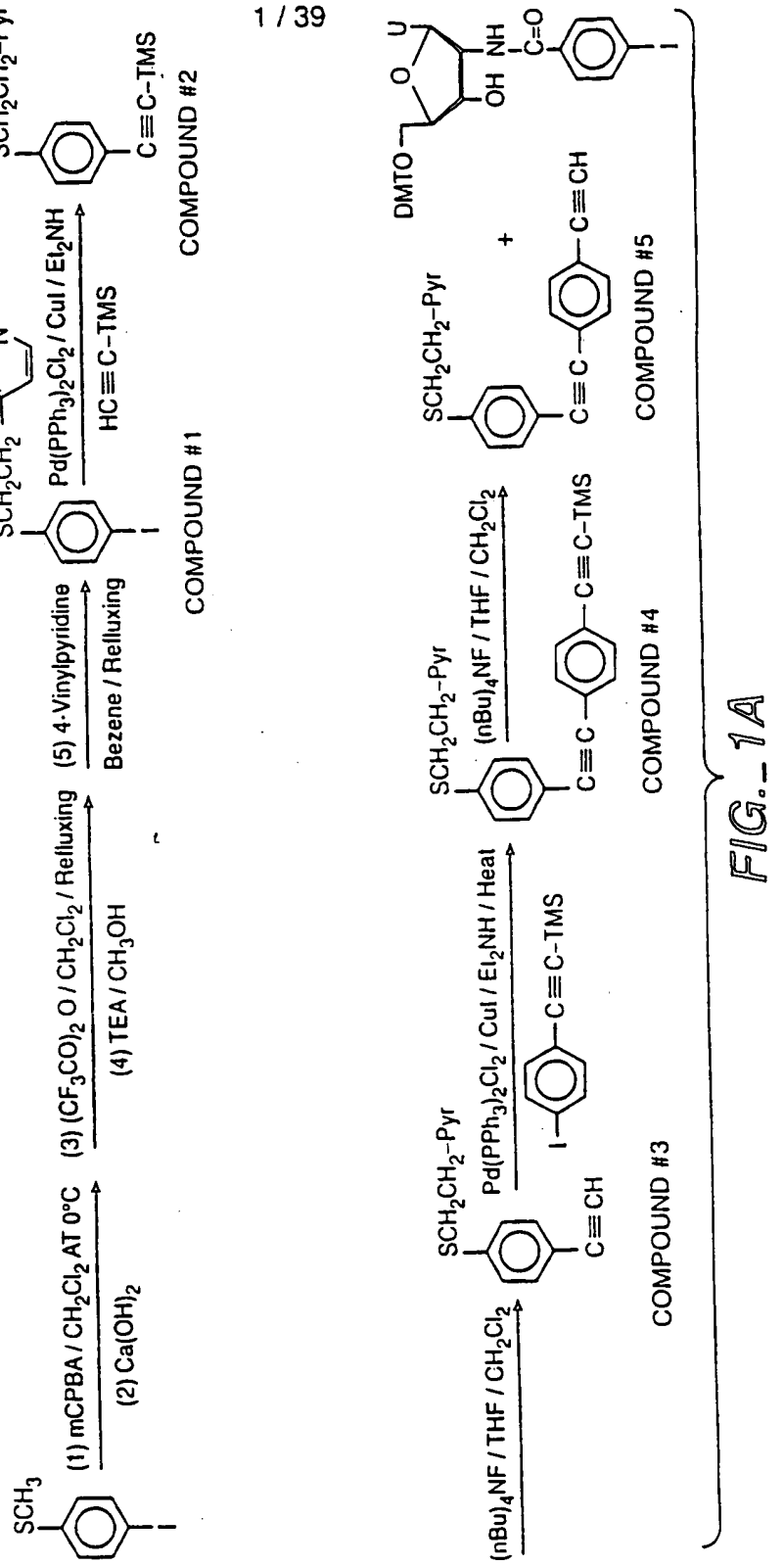

SCH3
(1) mCPBA / CH2Cl2 AT 0°C
(2) Ca(OH)2
(nBu)4NF / THF / CH2Cl2
SCH2CH2–Pyr
C≡CH
Pd(PPh3)2Cl2 / CuI / Et2NH / Heat
I
C≡C–TMS
COMPOUND #3
(3) (CF3CO)2 O / CH2Cl2 / Relluxing
(4) TEA / CH3OH
SCH2CH2–Pyr
C≡C
(nBu)4NF / THF / CH2Cl2
C≡C–TMS
COMPOUND #4
DMTO
O
U
OH
NH
C=O
SCH2CH2–Pyr
C≡C
C≡CH
COMPOUND #5
SCH2CH2
N
(5) 4-Vinylpyridine
Bezene / Relluxing
SCH2CH2–Pyr
Pd(PPh3)2Cl2 / CuI / Et2NH
HC≡C–TMS
C≡C–TMS
COMPOUND #1
COMPOUND #2

FIG._1A

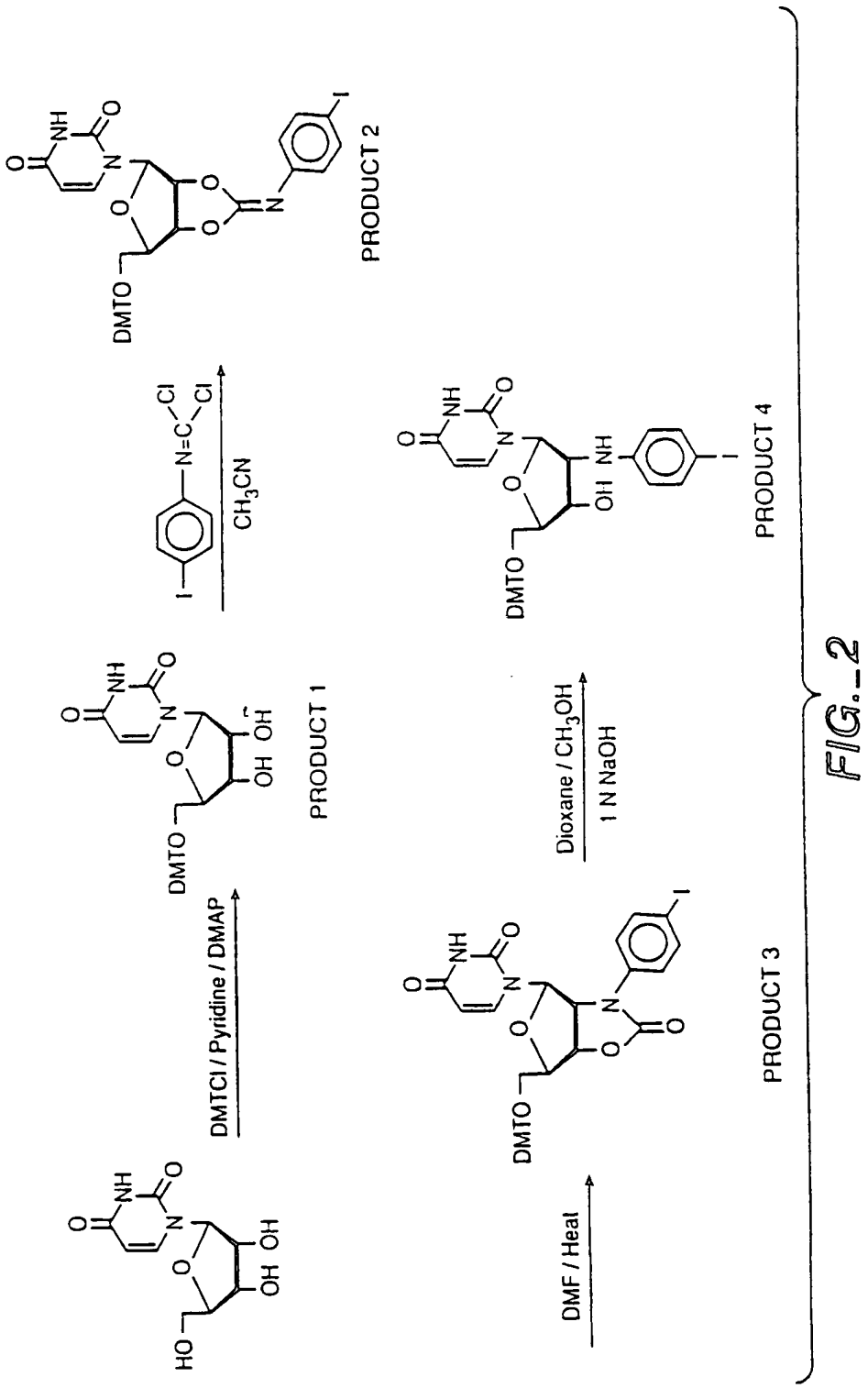


FIG._2

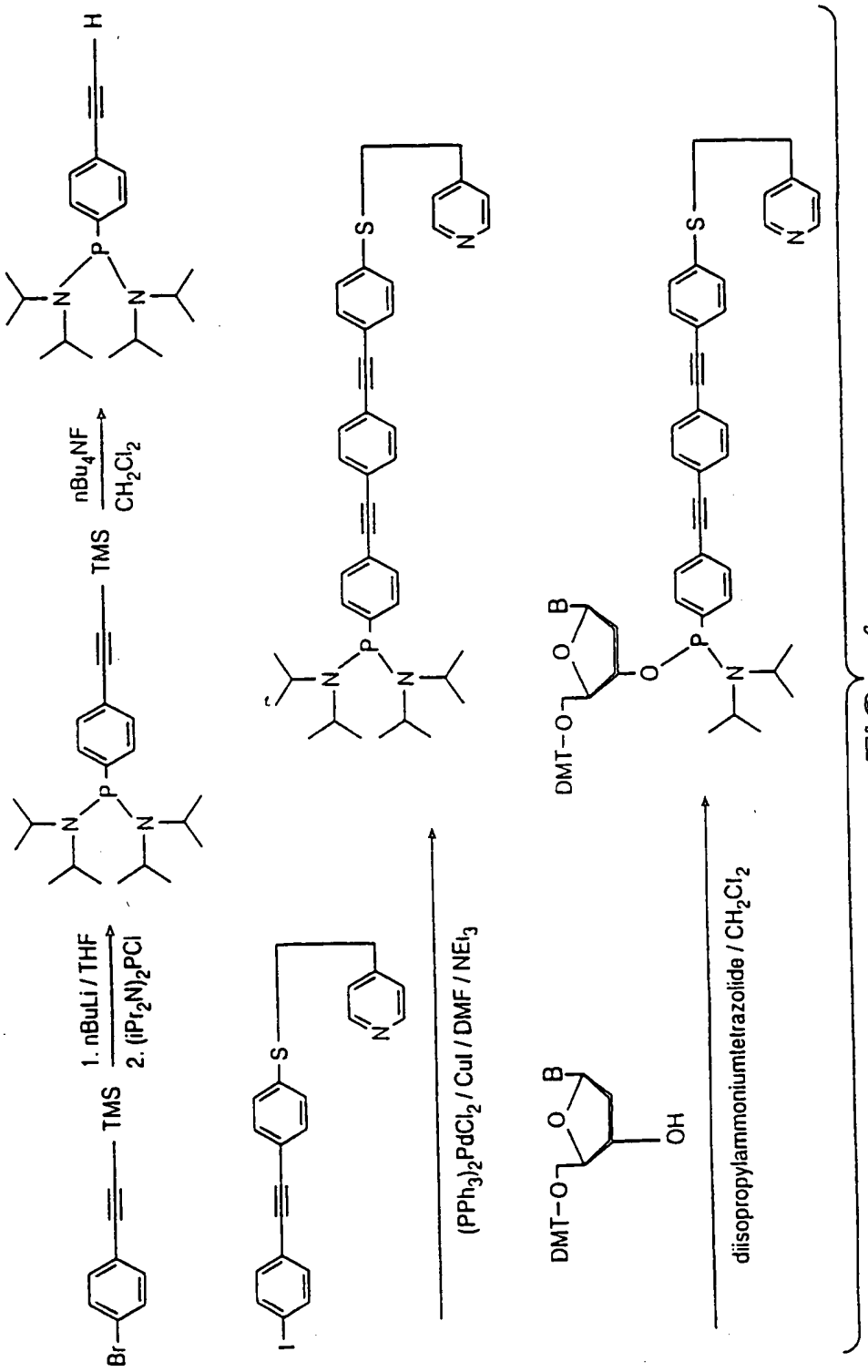


FIG._4

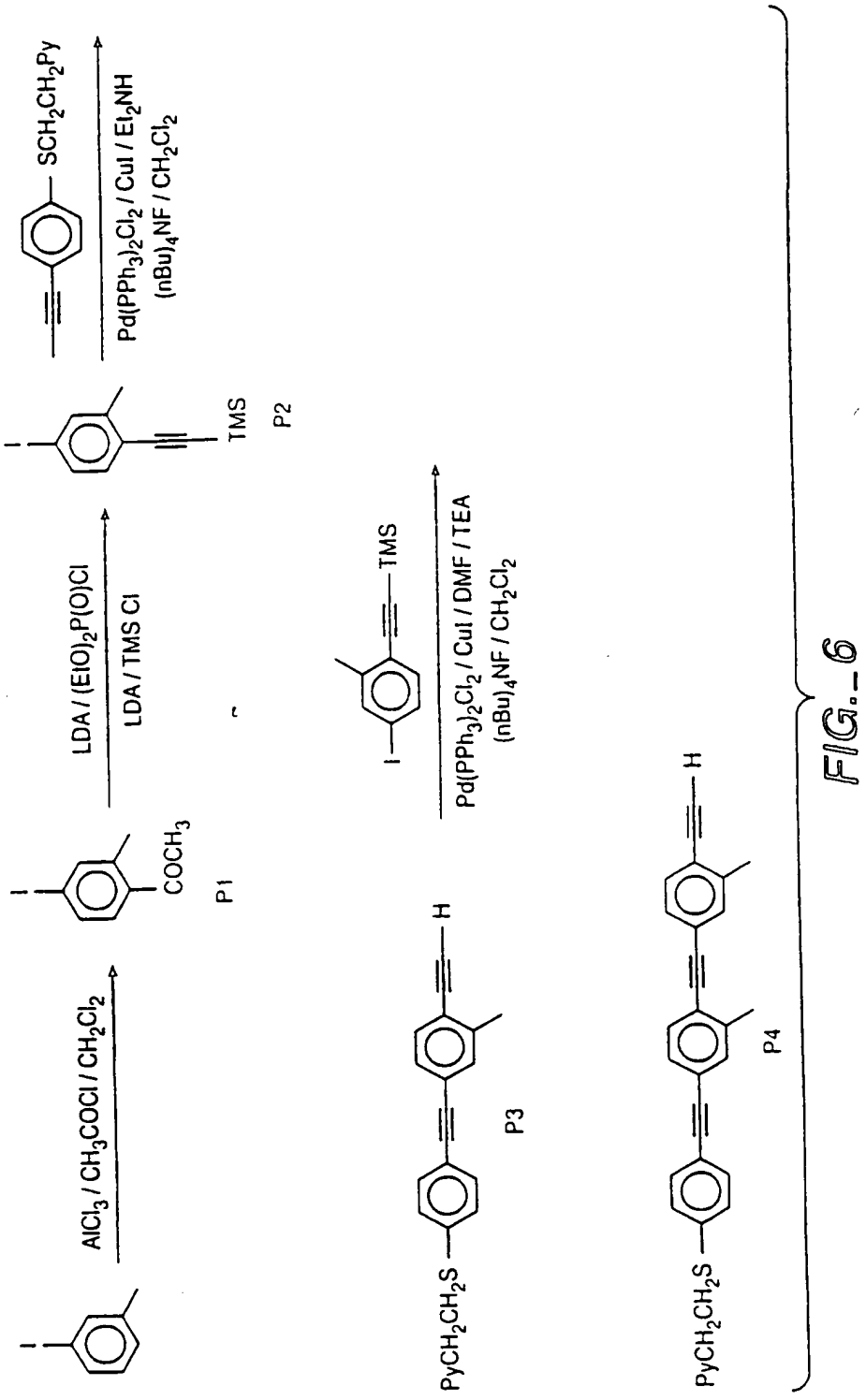


FIG._6

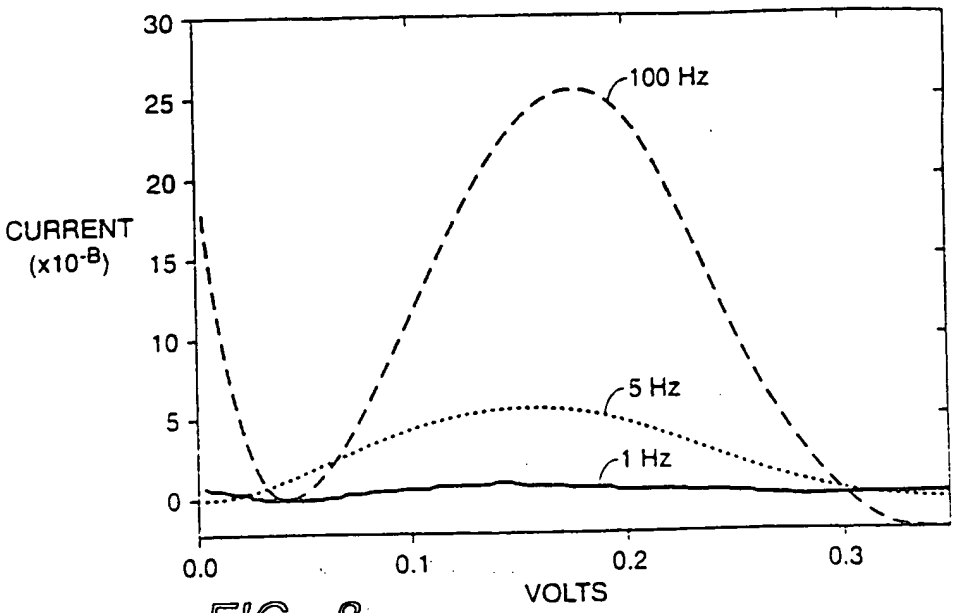


FIG._8

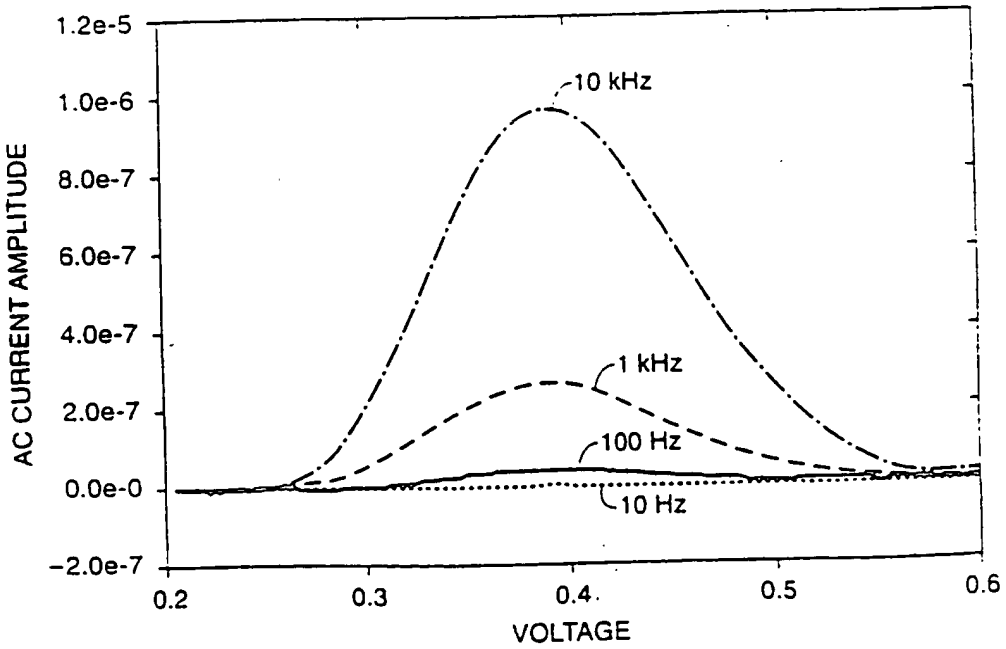


FIG._9A

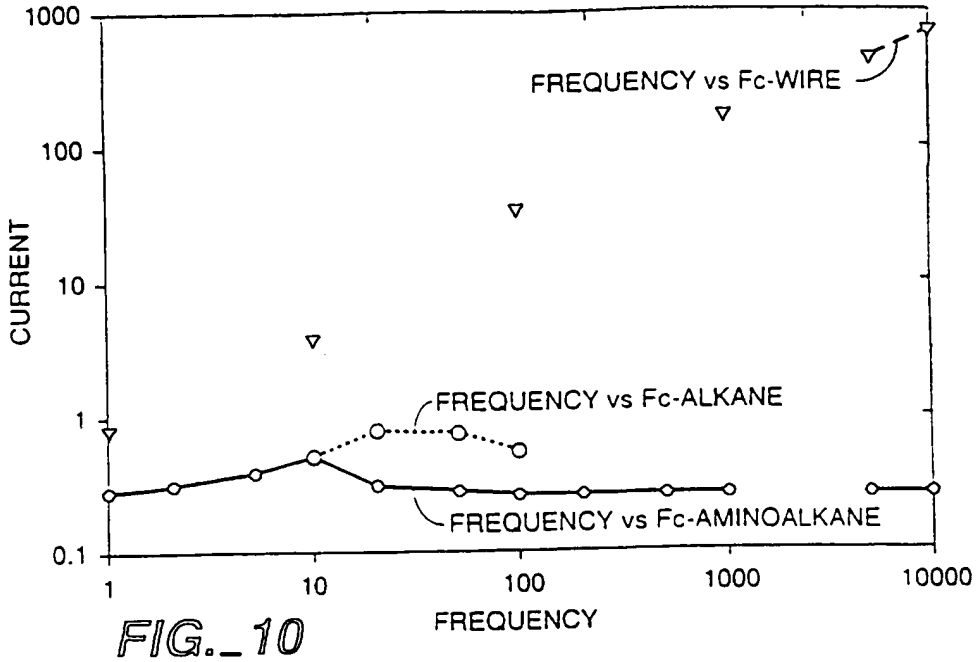


FIG._10

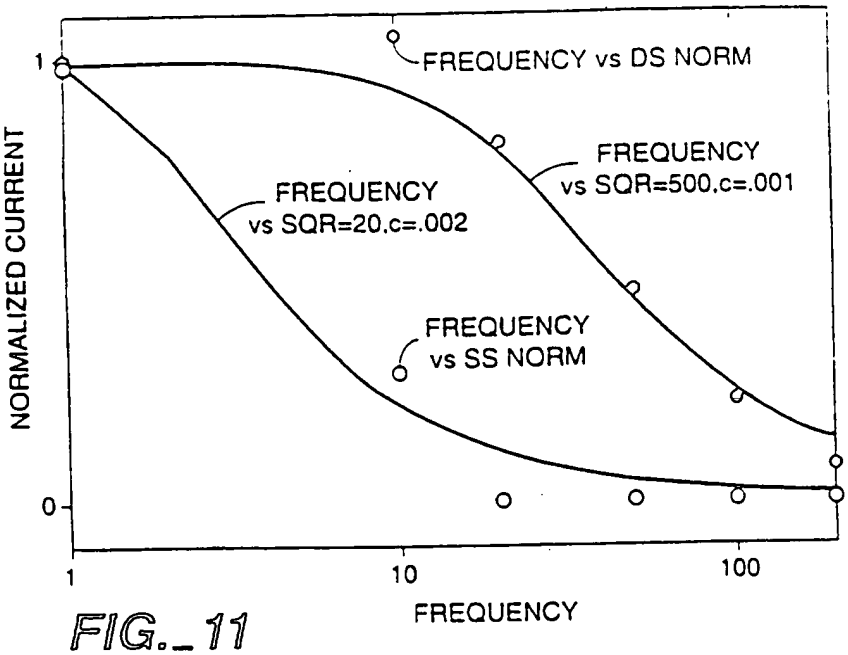


FIG._11

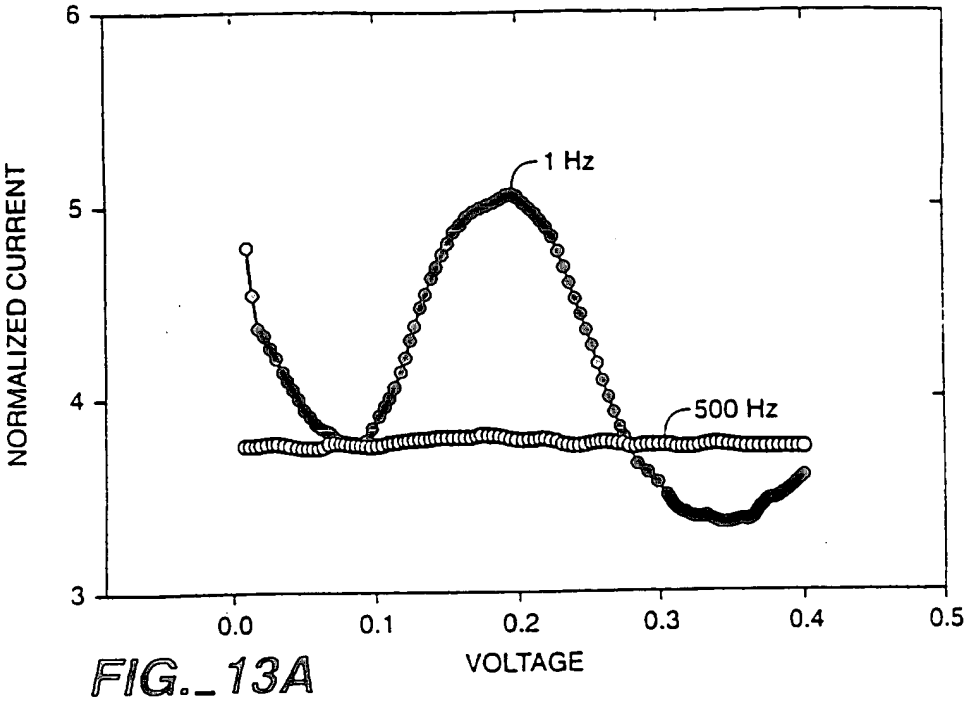


FIG._13A

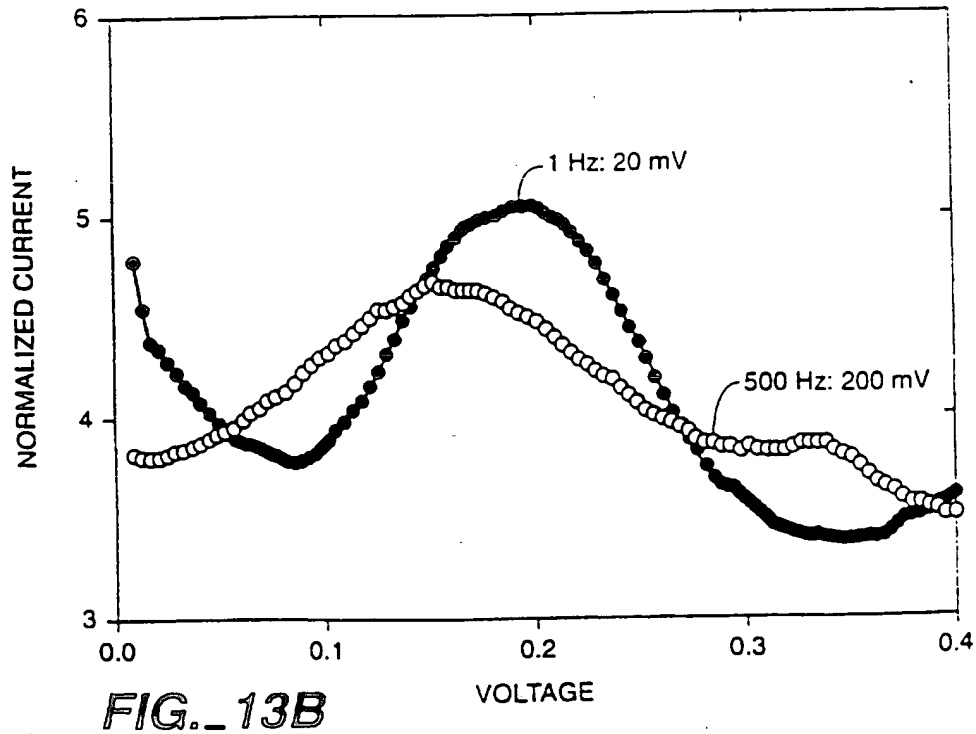


FIG._13B

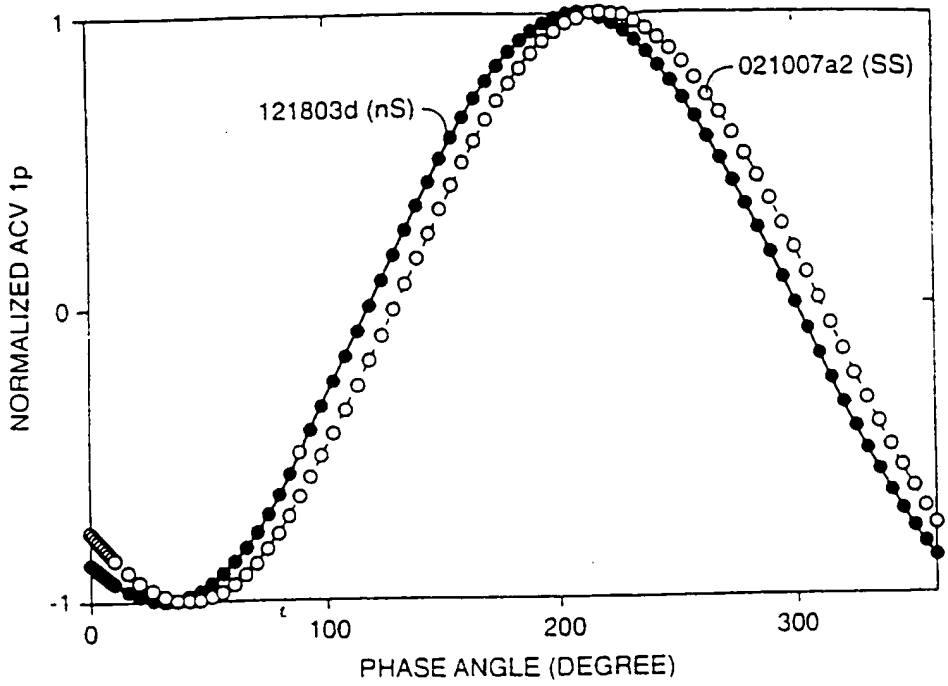


FIG._15B

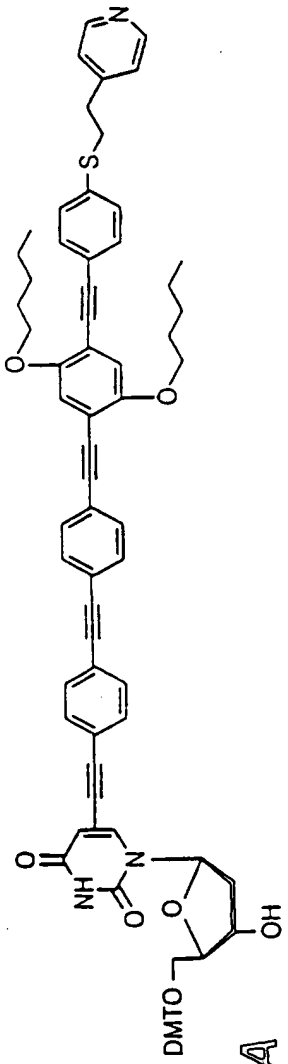


FIG._17A

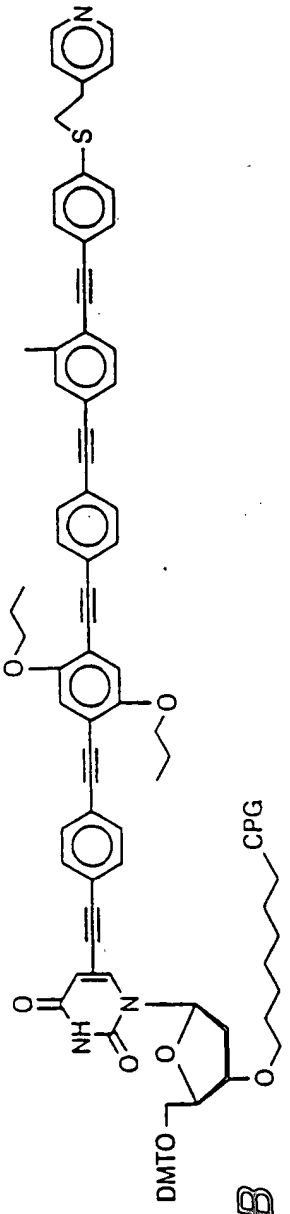


FIG._17B

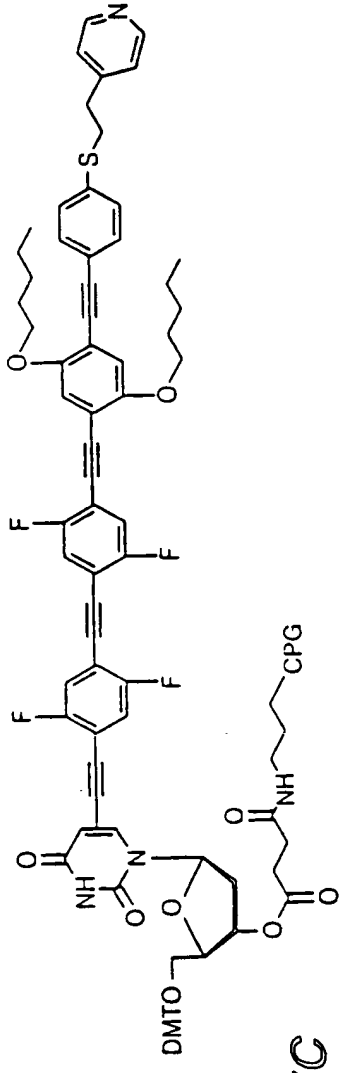
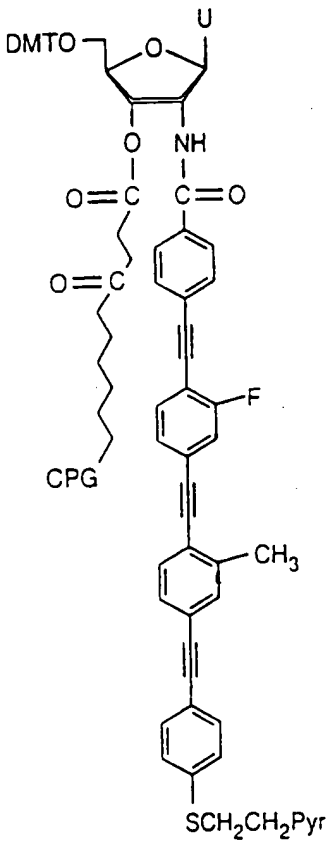
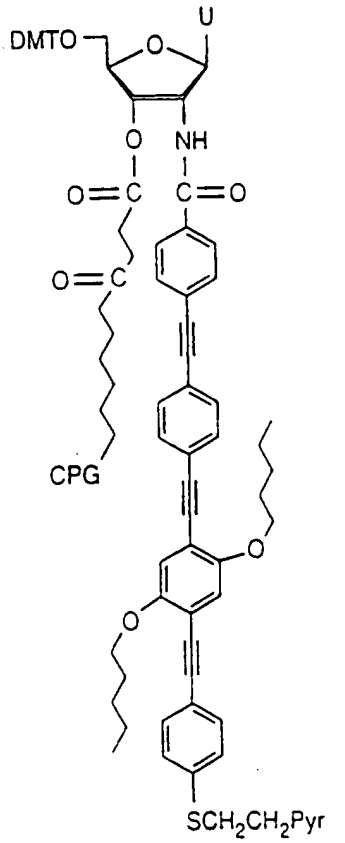


FIG._17C



4-BW-F-Uridine-CPG

FIG._17E



4-BW-2C5-Uridine-CPG

FIG._17F

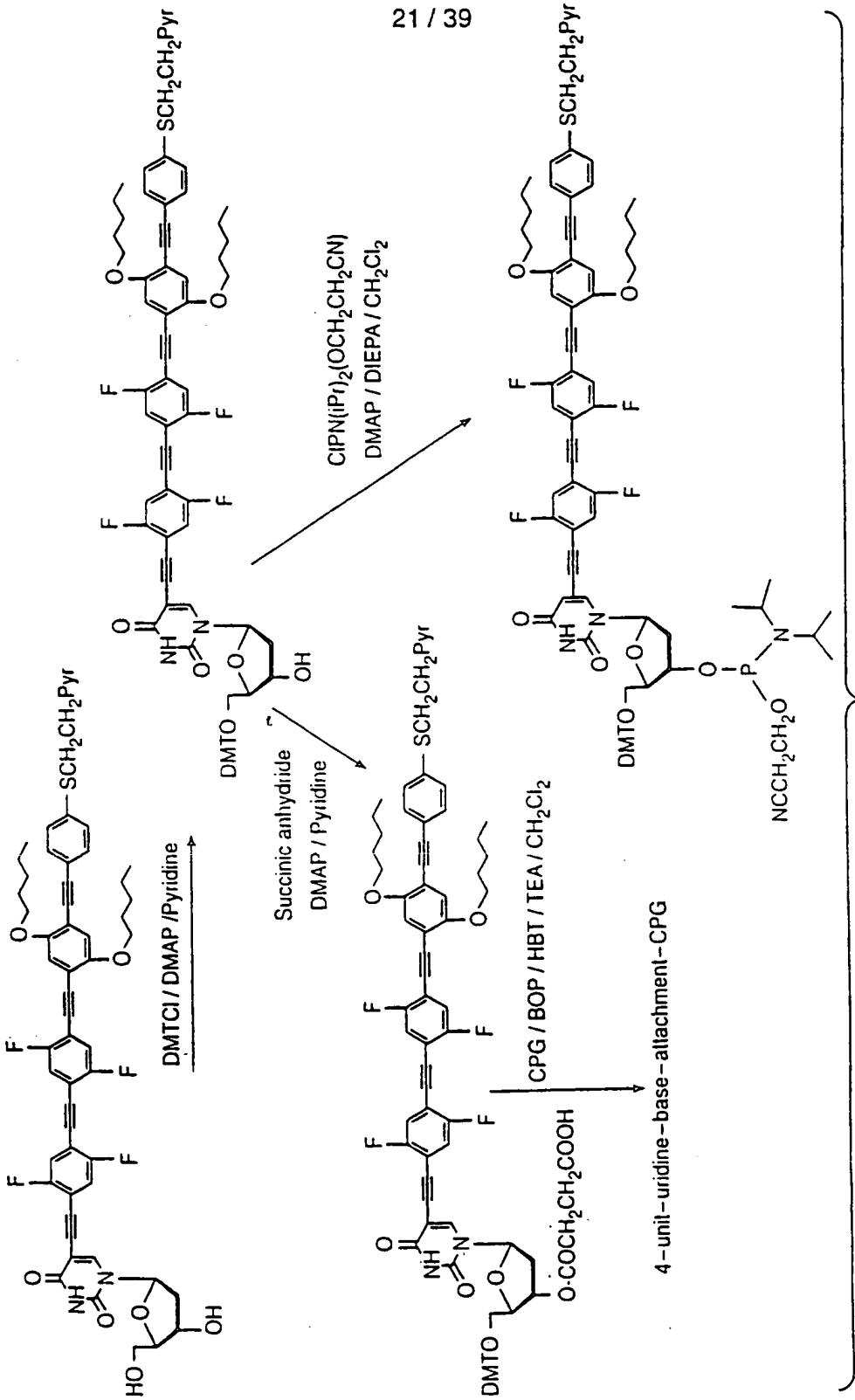


FIG._18B

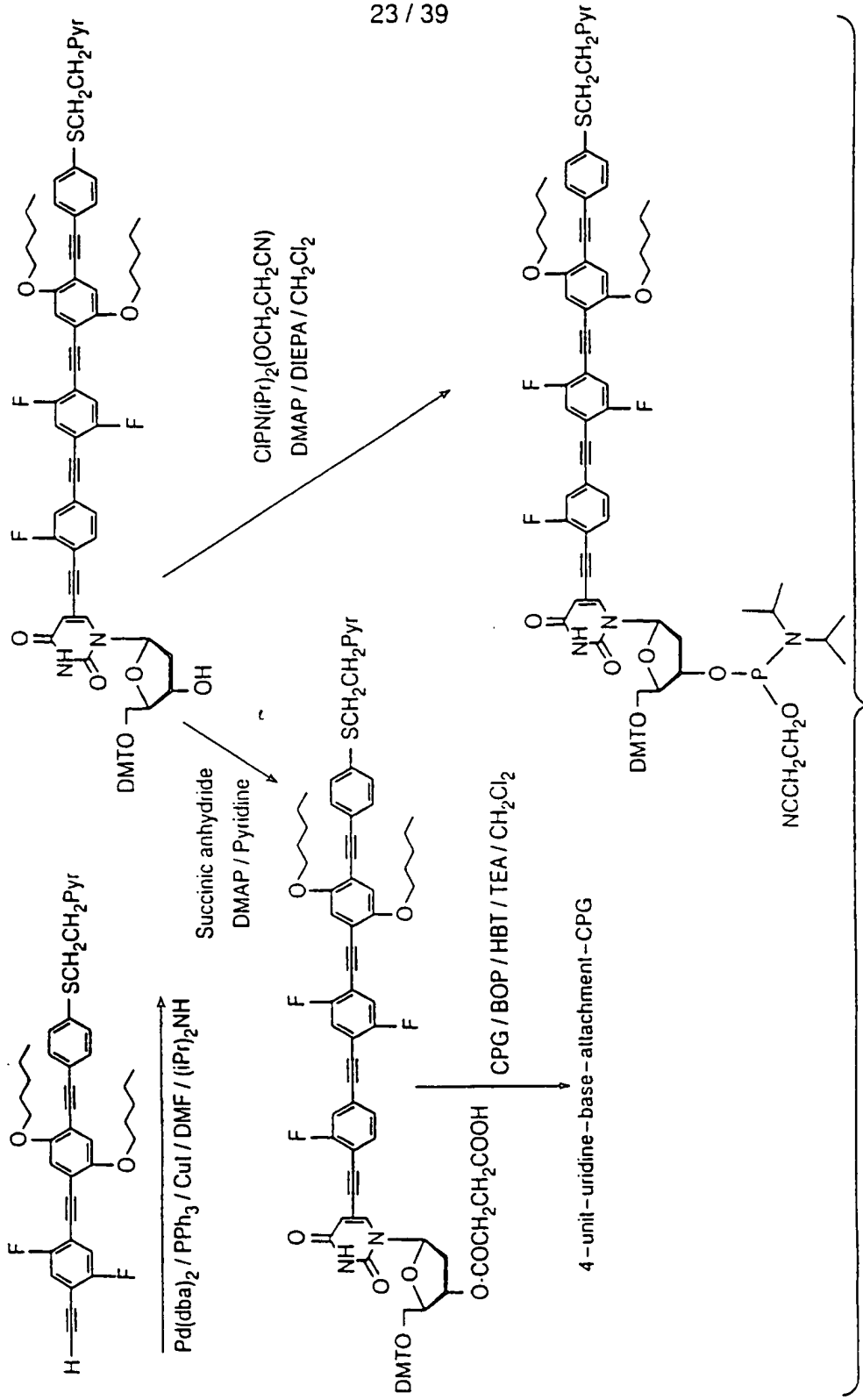


FIG. 19B

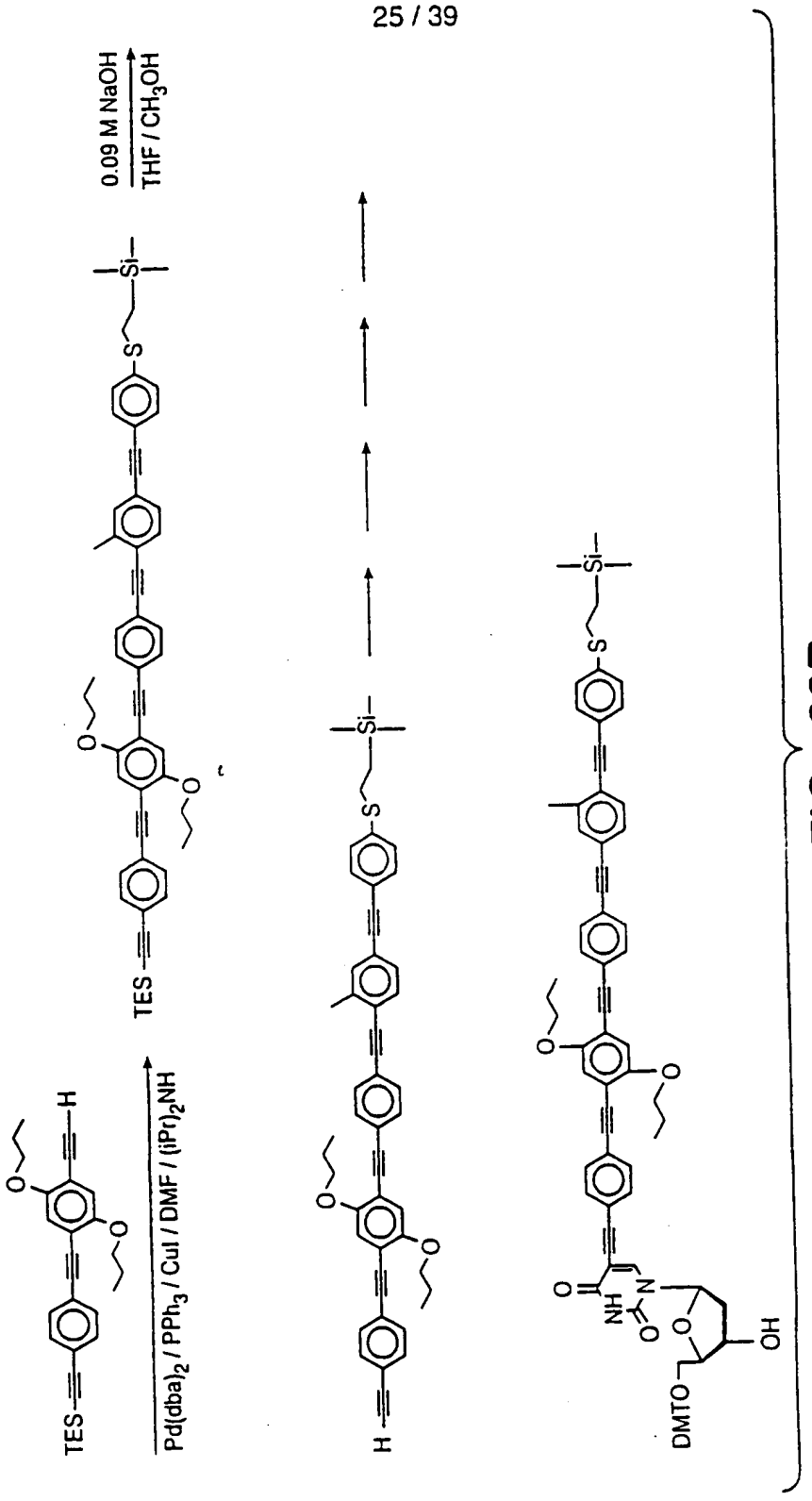


FIG._20B

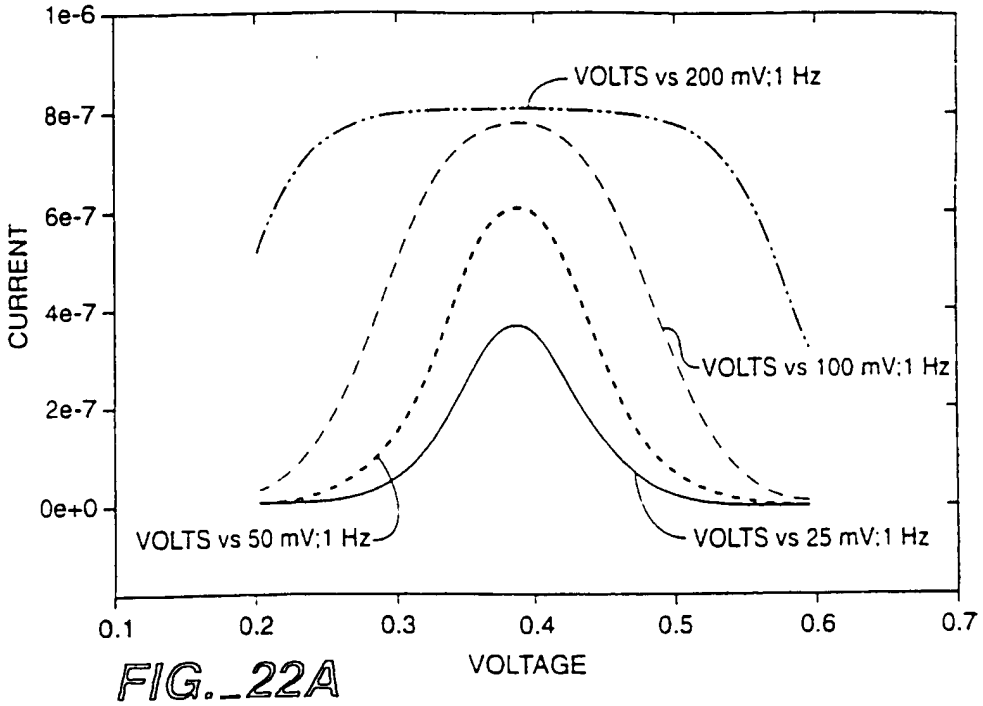


FIG._22A

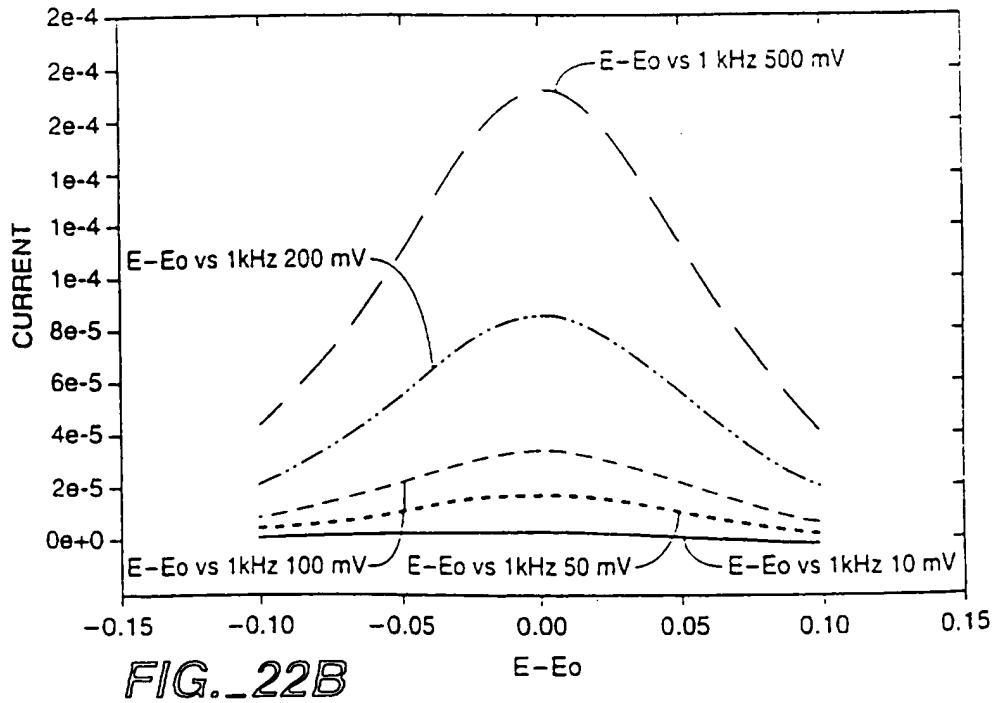


FIG._22B

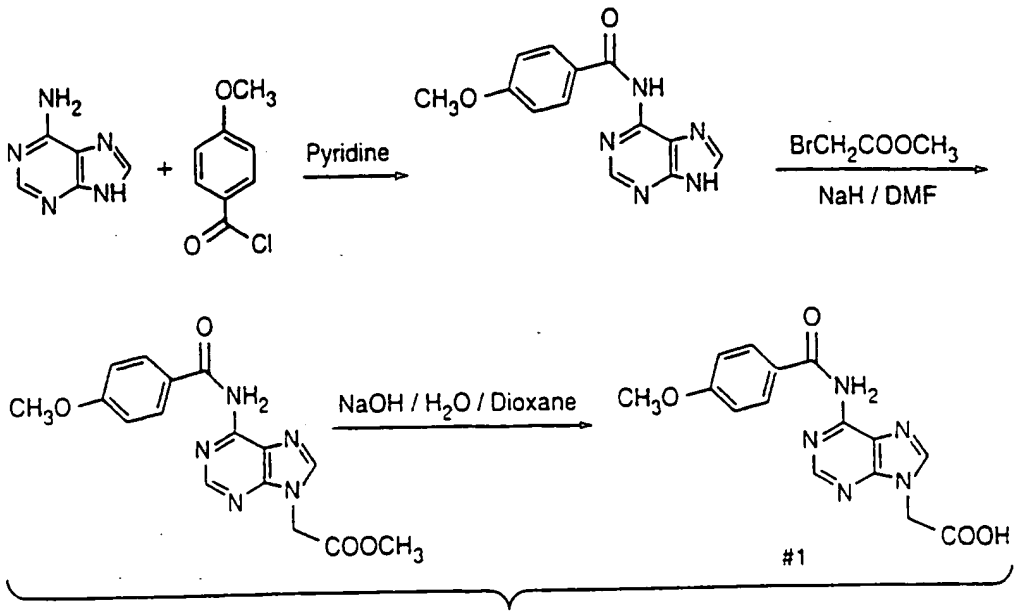

NH2
OCH3
Pyridine
CH3O
NH
BrCH2COOCH3
NaH / DMF
NaOH / H2O / Dioxane
COOCH3
COOH
#1

FIG._24

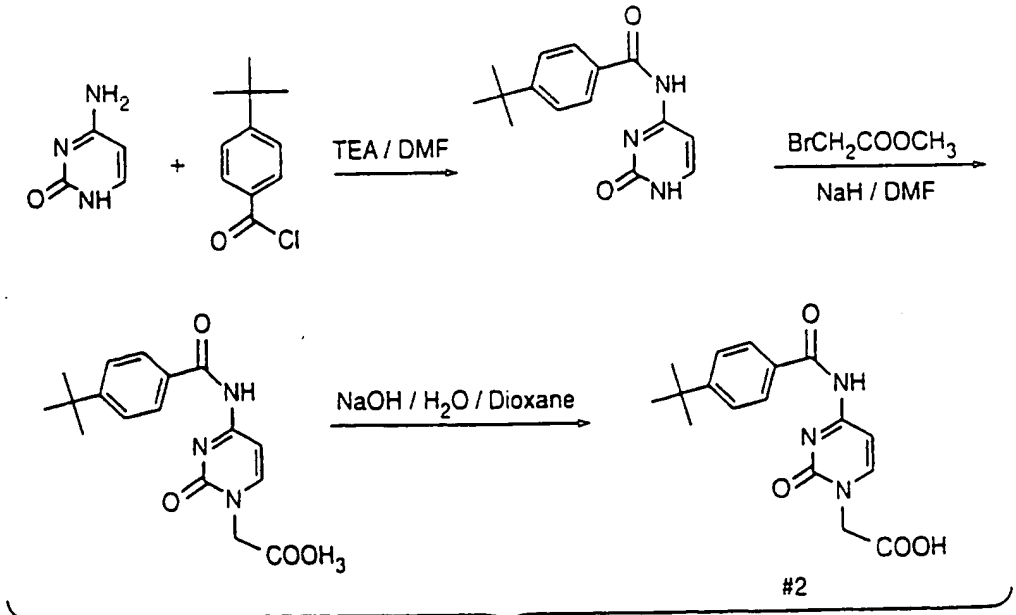

NH2
TEA / DMF
NH
BrCH2COOCH3
NaH / DMF
NaOH / H2O / Dioxane
COOH3
COOH
#2

FIG._25

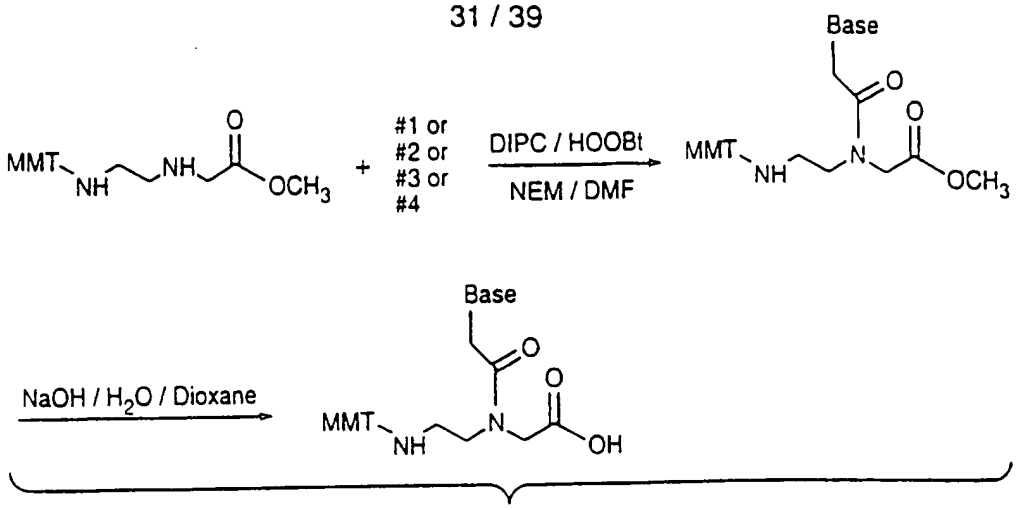
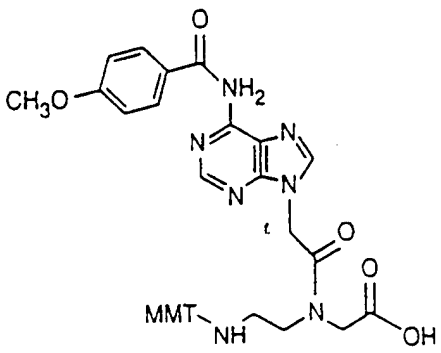
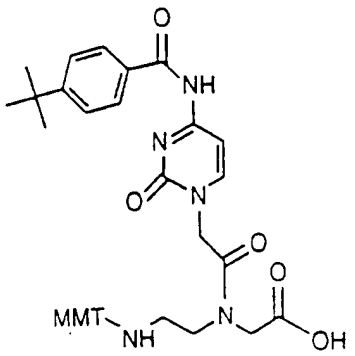


FIG._28A



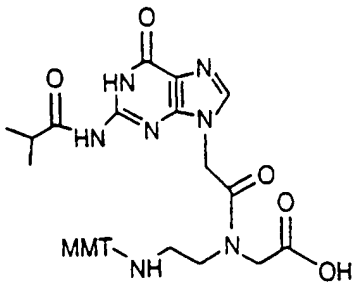
MMT-PNA-A

FIG._28B



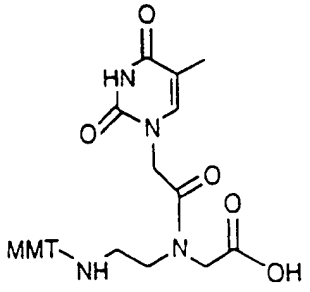
MMT-PNA-C

FIG._28C



MMT-PNA-G

FIG._28D



MMT-PNA-T

FIG._28E

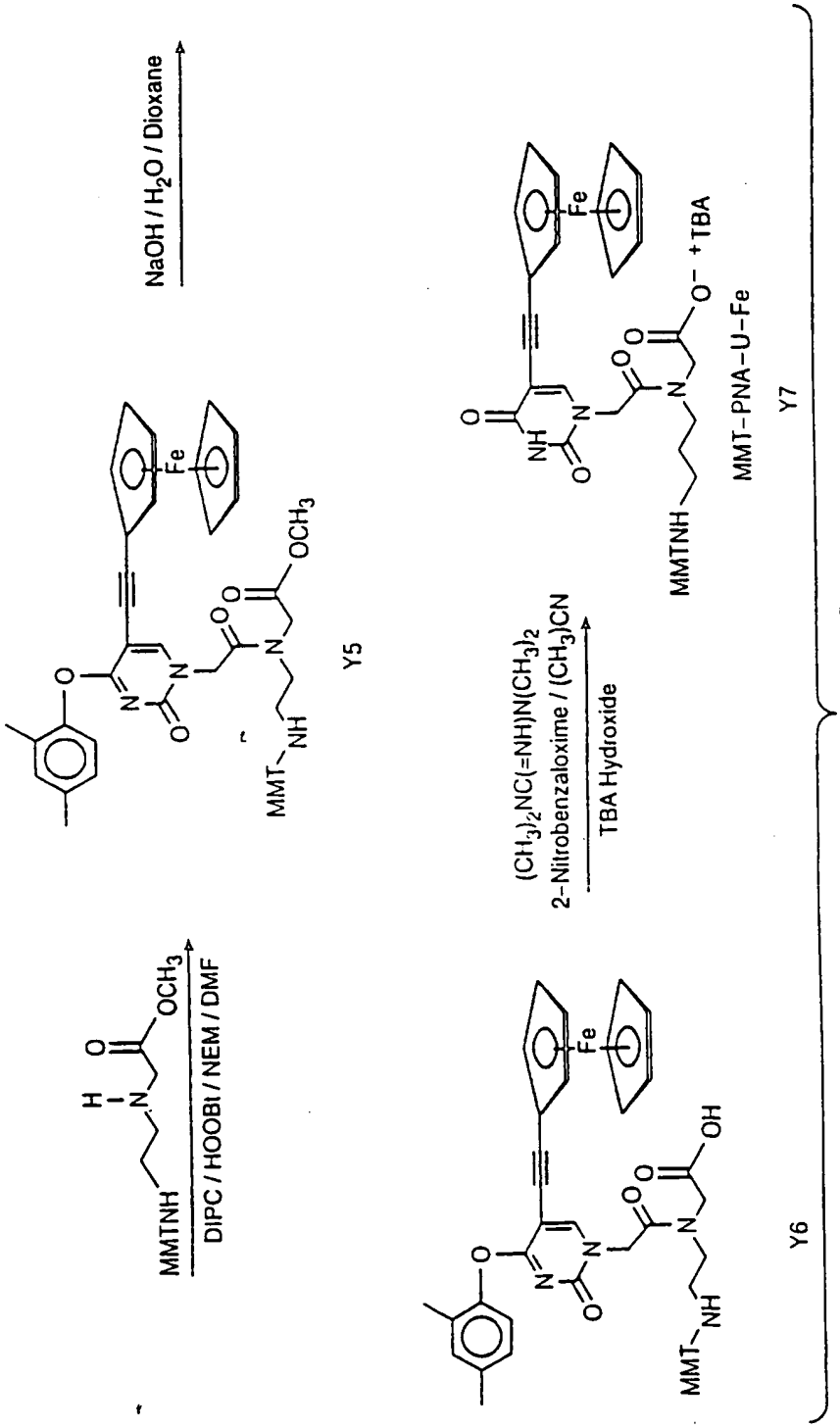

NaOH / H2O / Dioxane
DIPC / HOOBt / NEM / DMF
(CH3)2NC(=NH)N(CH3)2
2-Nitrobenzaloxime / (CH3)3CN
TBA Hydroxide
MMTNH
MMT
Fe
OCH3
OH
O- +TBA
Y5
Y6
Y7
MMT-PNA-U-Fe

FIG._29B

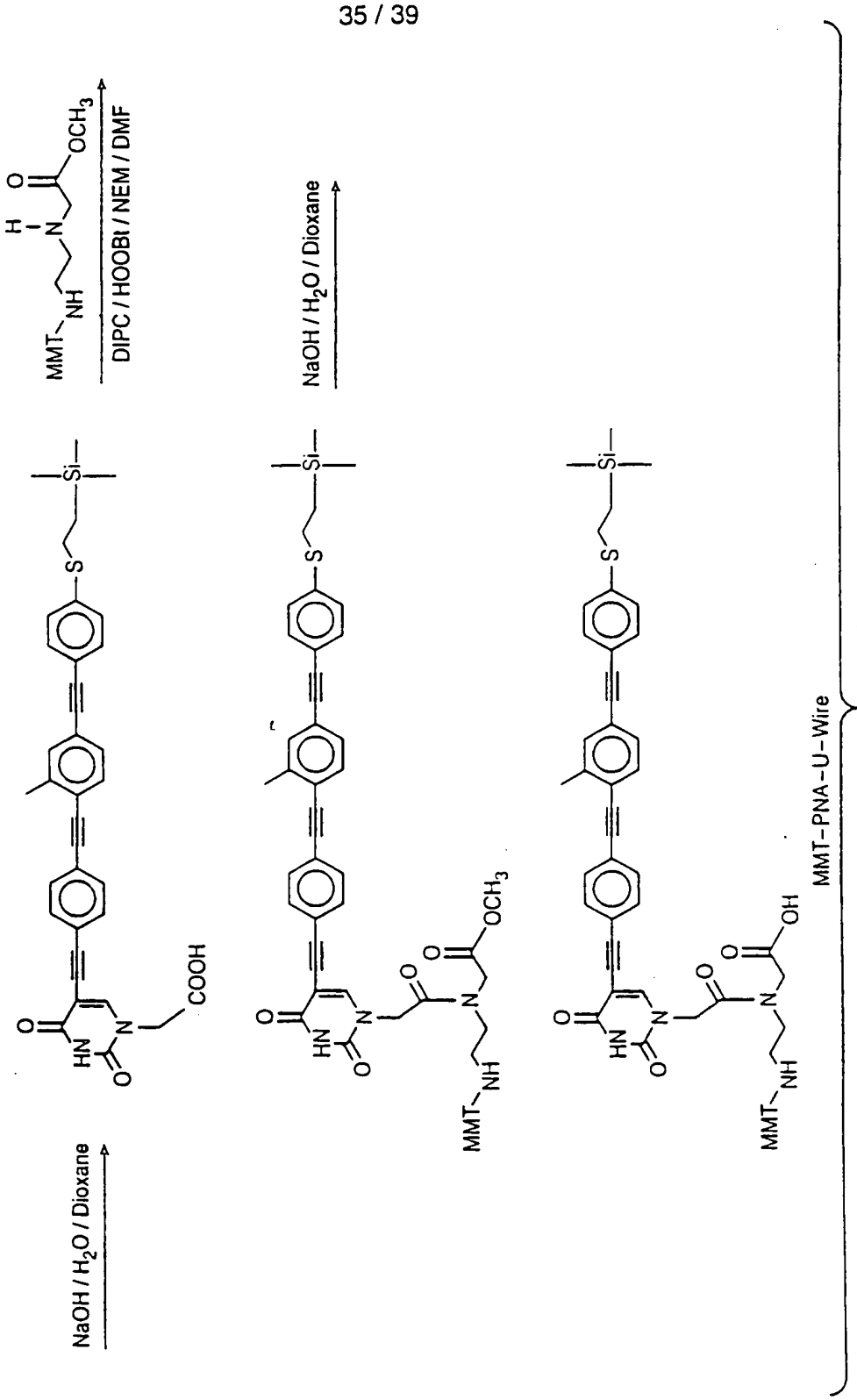


FIG._30B

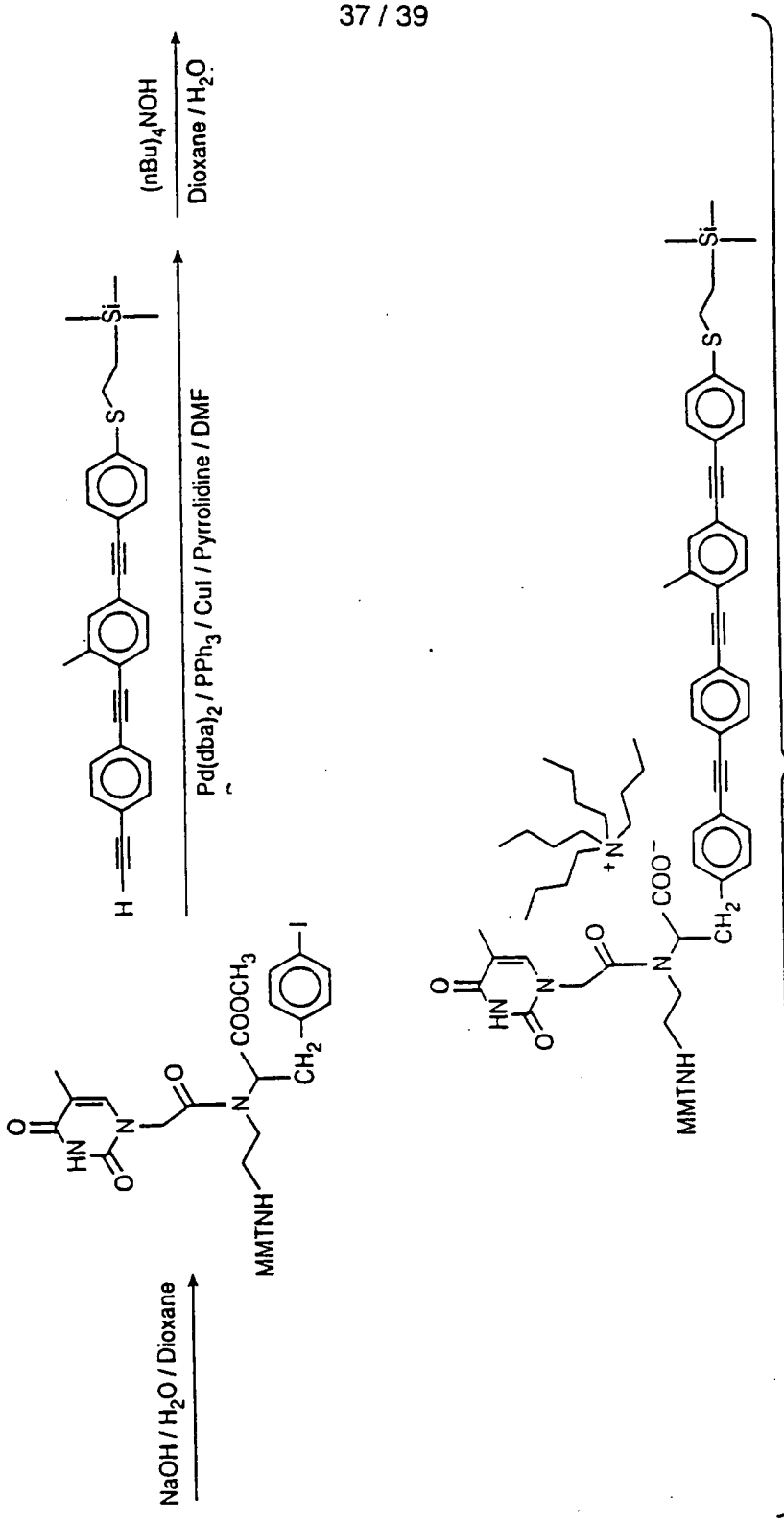


FIG._31B

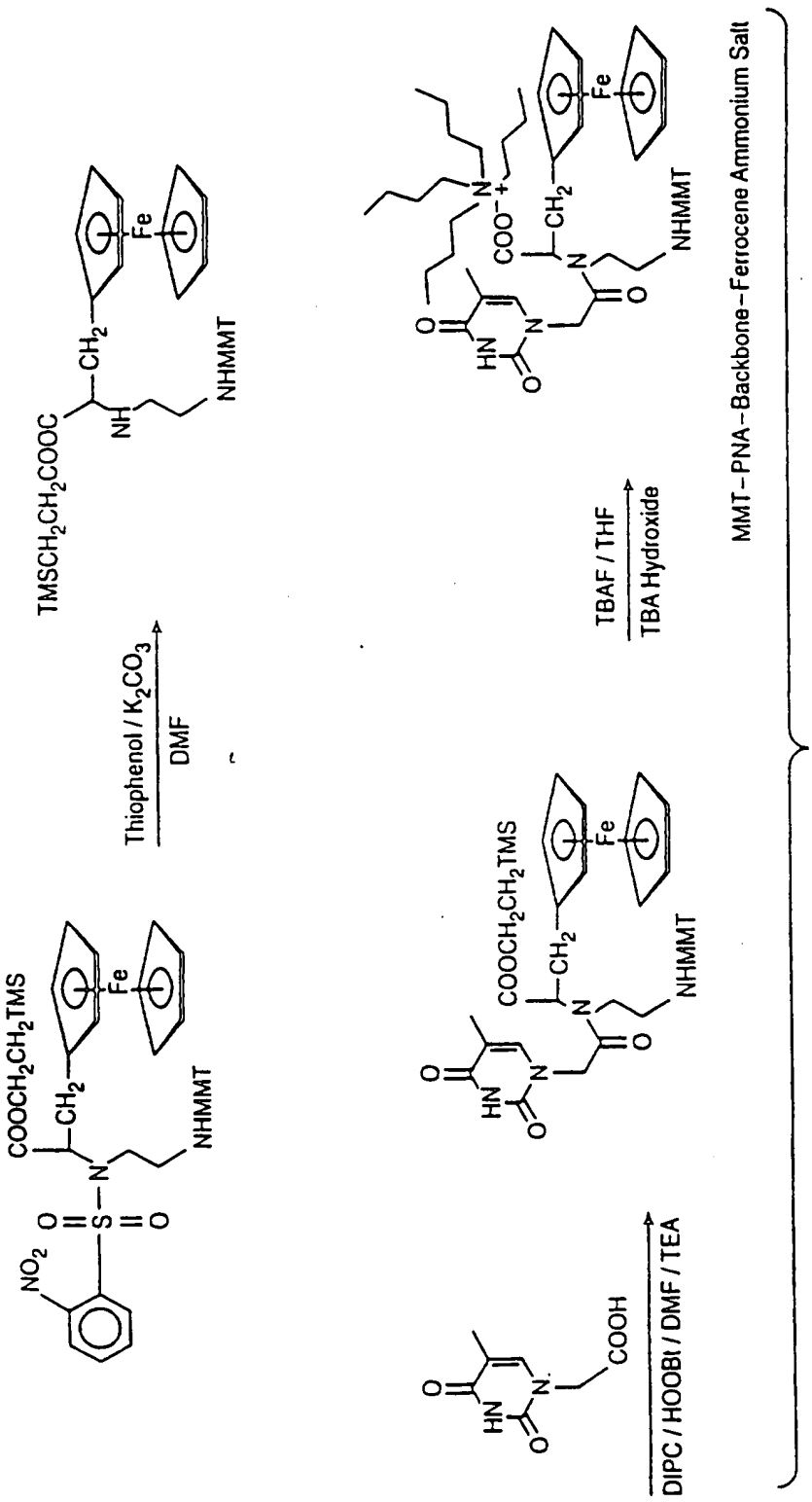


FIG._32B

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07H 21/00, C12Q 1/68
C07H 23/00

A3

(11) International Publication Number: WO 98/20162

(43) International Publication Date: 14 May 1998 (14.05.98)

(21) International Application Number: PCT/US97/20014

(22) International Filing Date: 5 November 1997 (05.11.97)

(30) Priority Data:

08/743,798	5 November 1996 (05.11.96)	US
60/040,155	7 March 1997 (07.03.97)	US
08/873,597	12 June 1997 (12.06.97)	US
08/873,978	12 June 1997 (12.06.97)	US
08/899,510	24 July 1997 (24.07.97)	US
08/911,085	14 August 1997 (14.08.97)	US
08/911,589	14 August 1997 (14.08.97)	US

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
12 November 1998 (12.11.98)

(54) Title: ELECTRODES LINKED VIA CONDUCTIVE OLIGOMERS TO NUCLEIC ACIDS

(57) Abstract

The invention relates to nucleic acids covalently coupled to electrodes via conductive oligomers. More particularly, the invention is directed to the site-selective modification of nucleic acids with electron transfer moieties and electrodes to produce a new class of biomaterials, and to methods of making and using them.

INTERNATIONAL SEARCH REPORT

Intr ional Application No
PCT/US 97/20014

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H21/00 C12Q1/68 C07H23/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07H C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 15971 A (CALIFORNIA INST OF TECHN) 15 June 1995 cited in the application see claims 1-20; figures 1-4; examples 1-7 ---	1-29
Y	R.P.HSUNG ET AL.: "Synthesis and CHaracterization of Unsymmetric Ferrocene-Terminated Phenylethynyl Oligomers." ORGANOMETALLICS, vol. 14, no. 10, 1995, pages 4808-4815, XP002077968 cited in the application see the whole document ---	1-29
Y	WO 93 10267 A (IGEN INC) 27 May 1993 see abstract; claim 1 ---	1-29
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
18 September 1998	01/10/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Scott, J

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INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 97/20014

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropnate, of the relevant passages	Relevant to claim No.
T	P.LINCOLN ET AL.: "Short-Circuiting the Molecular Wire." JOURNAL OF THE AMERICAN CHEMICAL SOCIETY., vol. 119, no. 6, 1997, pages 1454-1455, XP002077971 DC US see the whole document	1,9

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte 'onal Application No
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		WO 9515971 A	15-06-1995
		US 5770369 A	23-06-1998
		US 5780234 A	14-07-1998
		US 5705348 A	06-01-1998

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